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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/029,574	12/20/2001	Per Sonne Holm	3961.002	4954

7590 07/01/2004

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EXAMINER	
WHITEMAN, BRIAN A	

ART UNIT	PAPER NUMBER
1635	

DATE MAILED: 07/01/2004

Please find below and/or attached an Office communication concerning this application or proceeding.

Office Action Summary	Application No.	Applicant(s)	
	10/029,574	HOLM ET AL.	
	Examiner	Art Unit	
	Brian Whiteman	1635	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on 4/29/04.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) 1-20 is/are pending in the application.

4a) Of the above claim(s) 17-20 is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) 1-16 is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☒ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☒ All b)☐ Some * c)☐ None of:

1.☒ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☒ Information Disclosure Statement(s) (PTO-1449 or PTO/SB/08) Paper No(s)/Mail Date 4/17/02.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .
5) ☐ Notice of Informal Patent Application (PTO-152)
6) ☐ Other: _____.

DETAILED ACTION

Non-Final Rejection

Claims 1-20 are pending.

Election/Restrictions

Applicants' election with traverse of Group I (Claims 1-16) in Paper filed on 4/29/04 is acknowledged. The traversal is on the ground(s) that the examiner fails to show that examining all claims constitute a serious burden and applicant asserts that the claims of Group I and Group II concern the same essential invention because both are directed to replication of an E1-deificient adenovirus in a cell comprising YB-1 in its nucleus for the treatment of tumors, the groups only differ with respect to the source of YB-1. This is not found persuasive because as admitted by applicant the source of YB-1 is different for each method. The method in Group I requires transfecting a cell with an E1-deficient adenovirus comprising an YB-1 DNA sequence and expressing YB-1 in the cell and Group II does not require this method step. The method in Group I requires an E1-deficient adenovirus comprising an YB-1 DNA sequence and the method in Group II does not require the adenovirus. As stated in the election/restriction mails on 10/29/03, the search for each Group is not co-extensive and each group was classified in a different class/subclass. Other than applicants' assertion that the search for each group together will not be an undue burden on the examiner, the applicants have not provided sufficient evidence that it would not be an undue burden on the examiner to search both groups.

The requirement is still deemed proper and is therefore made FINAL.

Claims 17-20 are withdrawn from further consideration pursuant to 37 CFR 1.142(b), as being drawn to a nonelected invention, there being no allowable generic or linking claim. Applicant timely traversed the restriction (election) requirement in Paper filed on 4/29/04.

Priority

Receipt is acknowledged of papers submitted under 35 U.S.C. 119(a)-(d), which papers have been placed of record in the file.

Information Disclosure Statement

The applicants state that search reports were included in the IDS filed on 4/17/02. However, there are no search reports of record. If applicants want the search reports to be considered, then the search reports should be included in response to this office action.

Claim Rejections - 35 USC § 112

The following is a quotation of the first paragraph of 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

Claims 7-9 and 12 are rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the enablement requirement. The claim(s) contains subject matter, which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention.

Claims 10 and 11 are rejected under 35 U.S.C. 112, first paragraph, because the specification, while being enabling for a method of treatment for tumors comprising administering to a tumor in a patient in need thereof said E1 deficient adenovirus comprising a DNA sequence encoding YB-1 and administration of substances which damage tumor cells, surgical tumor excision, radiation therapy, chemotherapy, and hyperthermia, does not reasonably provide enablement for a method of treatment for tumors comprising administering to a patient in need thereof a medicament comprising an E1-deficient adenovirus comprising a YB-1 encoding DNA sequence and gene therapy. The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to use the invention commensurate in scope with these claims.

Claims 13-16 are rejected under 35 U.S.C. 112, first paragraph, because the specification, while being enabling for a method for E1-independent replication of replication defective adenovirus comprising administering to a tumor cell in vitro a recombinant adenovirus carrying a DNA sequence encoding YB-1, inducing expression of YB-1 in said tumor cell, causing said adenovirus to replicate in the presence of YB-1, does not reasonably provide enablement for a method for E1-independent replication of replication defective adenovirus comprising administering to a cell recombinant adenovirus carrying a YB-1 encoding DNA sequence, inducing expression of YB-1 in said cell. The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to use the invention commensurate in scope with these claims.

> Factors to be considered in determining whether a disclosure would require undue experimentation have been summarized in In re Wands, 858 F.2d 731, 8USPQ2d 1400 (Fed. Cir. 1988). They include (1) the quantity of experimentation necessary, (2) the amount of direction or guidance presented, (3) the presence or absence of working examples, (4) the nature of the invention, (5) the state of the prior art, (6) the relative skill of those in the art, (7) the predictability or unpredictability of the art, and (8) the breadth of the claims.

Applicants claim methods for treatment of tumors in a patent in need thereof, a method for E1 independent replication of a replication-defective adenovirus, wherein said methods use an E1 deficient adenovirus comprising a YB-1 encoding DNA sequence. In view of the guidance in the specification, the claimed methods are directed to a method of cancer gene therapy using the E1 deficient adenovirus.

At the time the application was filed, gene therapy was considered to be unpredictable due to significant problems in several areas. The state of the art, exemplified by Anderson et al., Nature, Vol. 392, pp. 25-30, 1998, displays major consideration for any gene transfer or any DNA therapy protocol involve issues that include:

1) The type of vector and amount of DNA constructs to be administered,

2) The route and time course of administration, the sites of administration, and successful uptake of the claimed DNA at the target site;

3) The trafficking of the genetic material within cellular organelles, the rate of degradation of the DNA, the level of mRNA produced, the stability of the mRNA product, the amount and stability of the protein produced, and

4) What amount of the expressed proteins considered to be therapeutically effective for a DNA therapy method.

In addition, all of these issues differ dramatically based on the specific vector used, the route of administration, the animal being treated, therapeutically effective amount of the DNA, and the disease being treated.

Anderson teaches that gene therapy is a powerful new technology that still requires several years before it will make a noticeable impact on the treatment of disease, and that several

major deficiencies still exist including poor delivery systems, both viral and non-viral, and poor gene expression after genes are delivered (pp. 25-30).

Anderson further teaches that the reason for the low efficiency of gene transfer and expression in human patients is that we still lack the basis understanding of how vectors should be constructed what regulatory sequences are appropriated for which cell types (page 30, column 1, last paragraph). Furthermore, Verma, *Nature*, Vol. 389, pages 239-242, 1997, indicates that factors including the nature of the diseases and/or disorders, the nature of a DNA and/or target tissue, and a delivery system and/or amounts of the DNA complexes employed in the delivery system that would generate a therapeutic effect *in vivo* must be considered for any gene therapy method to be successful (page 238, columns 1 and 2).

In further view of the doubts expressed above by Anderson and Verma, the state of the art for cancer gene therapy as discussed by Vile et al., (Gene Therapy, Vol. 7, pp. 2-8, 2000). Vile teaches:

> The problems which gene therapy for cancer will take into the next millennium focus far less on the choice of therapeutic gene(s) to be used than on the means of delivering them. There is already a battery of genes that we know are very effective in killing cells, if they can be expressed at the right site and at appropriate levels. None the less, until the perfect vector is developed, the choice of gene will remain crucially important in order to compensate for the deficiencies of the vectors we currently have available (page 2, 1st paragraph, left column). Whatever its mechanism, no single genes can be a serious contender unless it has a demonstrable bystander effect (page 2, right column). The requirement for such a bystander effect stems directly from the poor delivery efficiency provided by current vectors (page 2, right column).
>
> A genuine ability to target delivery systems to tumor cells distributed widely throughout the body of a patient would simultaneously increase real titers and efficacy. In truth, no such systemically targeted vectors exist yet. Injection of vectors into the bloodstream for the treatment of cancer requires not only that the vectors be targeted (to infect only tumor cells) but also that they by protected (from degradation, sequestration or immune attack) for long periods of time so that they can reach the appropriate sites for infection. Moreover, having reached such sites, the vectors must be able to penetrate into the tumor

> from the bloodstream before carrying out their targeted infection (page 4, bottom left column and top right column).

Thus, at the time the application was filed, the state of the art for gene therapy was considered highly unpredictable.

For additional reviews of the unpredictability of the gene therapy art, see Gomez-Navarro et al., European Journal of Cancer, Vol. 35, pp. 867-885, 1999; McNeish et al., Gene Therapy, pp. 1-7, 2004; Green et al., Cancer Gene Therapy, 9:1036-1042 2002; Alemany et al., Nature Biotechnology, 18:723-727, 2000; Gromeier, ASM News, 68:438-445, 2002.

With respect to the treatment methods in claims 7-12, the specification is only enabled for combination cancer therapy comprising administering said E1 deficient adenovirus comprising YB-1 encoding DNA sequence and administration of substances which damage tumor cells, surgical tumor excision, radiation therapy, chemotherapy, hyperthermia and not for the full breadth of the claimed method because it would have taken one skilled in the art an undue and excessive amount of experimentation to practice using an E1 deficient adenovirus by itself to treat a tumor in a patient. The art of record teaches that cancer gene therapy is unpredictable. The unpredictability taught by the art of record involves poor and inefficient delivery of adenovirus to target a tumor, host immune response which limit the ability of the adenovirus to infect a tumor, failure to efficiently infect certain tumors which lack adenoviral receptor CAR, promiscuous tropism which causes uncontrolled adenoviral infection and gene transfer into normal bystander cells, uptake intake into the liver of adenovirus instead of uptake into target tumor when the virus is systemically (e.g., intravenous administration) delivered to a patient.

With regard to previous experience with adenovirus to treat cancer, McNeish et al., (supra) teaches that in 93 patients receiving adenoviral particles, no objective clinical response were seen patients receiving the virus alone but that some responses were seen in patients receiving the virus in combination with chemotherapeutic agents. McNeish further teaches that:

Although targeting tumor suppressor gene pathways is an attractive and logical strategy for cancer gene therapy, results from clinical trials have not mirrored the preclinical studies. Clearly, the ability to induce cell cycle arrest and apoptosis in vitro or growth arrest in mouse xenografts does not guarantee response in clinical trials. See page 5.

This is further supported by Gomes-Navarro et al., (supra), who teaches that, "the spontaneous behavior of human tumors is somewhat different for that of malignant cells in vitro, and from that of experimental tumors in animal models."

Applicants provide no working example of the methods set forth in claims 7-12. The applicants teach that an E1 minus adenovirus coding for YB-1 can kill tumor cells in vitro; however, the art of record and the specification do not teach one skilled in the art how to correlate between results obtained in vitro studies set forth in the specification with results which the skilled artisan would reasonably expect to see in vivo. Furthermore, oncolysis in a cell line does not provide a nexus to treatment of tumors in vivo because the art of record and the specification do not provide sufficient guidance and/or factual evidence that killing tumor cells in vitro reasonably extrapolates to treatment of a tumor in vivo because killing tumor cells in vitro does not indicate that the number of tumor cells killed in a tumor in vivo is more than the number of new tumor cells in the tumor.

Furthermore, with respect to claim 10 directed to using additional therapy with the claimed E1 deficient adenovirus, the specification provides sufficient guidance for a method of treating a tumor in a patient comprising administering said E1 deficient adenovirus comprising

YB-1 encoding DNA sequence and administration of substances which damage tumor cells, surgical tumor excision, radiation therapy, chemotherapy, and hyperthermia because the additional methods listed in claim 10 were already known in the art, at the time the application was filed, for treating a tumor in a patient. In addition, the specification and the art of record do not provide sufficient guidance and/or factual evidence that the claimed adenovirus would counteract the additional therapies set forth in claim 10. However, claim 10 reads on using gene therapy in combination with the claimed E1 deficient adenovirus. The art of record and the specification are absent for using gene therapy in the claimed method. The relevance of using gene therapy is unclear because neither the applicants nor the prior art teach a nexus between using the claimed method and using gene therapy in the claimed method.

Given the above analysis of the factors, it is concluded that the specification provides sufficient guidance for combination therapy comprising administering said E1 deficient adenovirus comprising YB-1 encoding DNA sequence and administration of substances which damage tumor cells, surgical tumor excision, radiation therapy, chemotherapy, and hyperthermia and not for the full scope of the claimed invention.

Furthermore, with respect to claims 13-16, the claims can read on a method for E1-independent replication of a replication defective adenovirus in vitro or in vivo. With regard to the claimed method practiced in vitro, applicants' disclosure does teach one skilled in the art how to use this method on tumor cells in vitro. The in vitro embodiments involve transfecting of immortalized cells with the adenovirus showing nucleus location of YB-1 using E1-deficient adenovirus encoding YB-1 DNA sequence; showing oncolysis of tumor cell lines by adenovirus expressing YB-1; and showing formation of adenoviral particles in a cancer cell line.

In addition, with respect to using the claimed methods in vivo, the only disclosed use for in vivo is for treatment of tumors, cancers, malignant diseases, cells and tissues exhibiting aberrant growth. For the reasons set forth above, the claimed in vivo method is not considered enabled. For the unpredictability of cancer gene therapy see Vile (supra), Green (supra) and Gomes-Navarro (supra). Applicants provide no working example of the in vivo method embraced in claims 13-16. The applicants teach that an E1 minus adenovirus coding for YB-1 can kill tumor cells in vitro. In view of the In Re Wands Factors, one skilled in the art could correlate not from the results obtained from the in vitro studies to killing tumor cells in vivo using administration of the claimed adenovirus. In addition, the specification does not teach how to overcome the problems taught by the art of record with adenovirus gene therapy and using any route of administration. Therefore, it would take one skilled in the art an undue amount of experimentation to determine what route of administration (*e.g.* intravenous, dermal, nasal, rectal, vaginal, inhalation, or topical administration) would result in oncolysis of a tumor cell in vivo using the claimed methods.

In conclusion, the as-filed specification and the claims coupled with the art of record, at the time the invention was made, only provide sufficient guidance and/or evidence to reasonably enable a method for E1-independent replication of replication defective adenovirus comprising administering to a tumor cell in vitro a recombinant adenovirus carrying a DNA sequence encoding YB-1, inducing expression of YB-1 in said tumor cell, causing said adenovirus to replicate in the presence of YB-1 and not for the full breadth of the claimed invention. Given that cancer gene therapy wherein an adenovirus is employed to treat a tumor in an individual was unpredictable at the time the invention was made, and given the lack of sufficient guidance as to

a cancer gene therapy effect produced by any adenovirus cited in the claims, one skilled in the art would have to engage in a large quantity of experimentation in order to practice the claimed invention based on the applicants' disclosure and the unpredictability of cancer gene therapy.

Claim Rejections - 35 USC § 102

The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless –
>
> (f) he did not himself invent the subject matter sought to be patented.

Claims 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, and 11 are rejected under 35 U.S.C. 102(f) because the applicant did not invent the claimed subject matter. U.S. application 10/451,210 has one common inventor with the instant application. In addition, at the time the instant application was examined there was no evidence of record that the application and US 10/451,210 were commonly assigned.

Claims 1-6 are directed to an E1-deficient adenovirus comprising a DNA sequence encoding a YB-1 protein. US '210 claims an adenoviral nucleic acid comprising a nucleic acid sequence coding for YB-1, wherein the adenoviral nucleic acid is E1 deficient (claim 53). El deficient means that the adenovirus is E1a and E1b deficient. US '210 claims an adenovirus comprising the adenoviral nucleic acid (claim 65).

Claims 7-11 embrace using combination therapy comprising administering said E1 deficient adenovirus comprising a DNA sequence encoding YB-1 and administration of substances which damage tumor cells, surgical tumor excision, radiation therapy, chemotherapy,

and hyperthermia. US '210 claims using a cyostatic agent with the adenovirus to treat a tumor disease (claims 68, 70, and 71).

Double Patenting

The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. See *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970);and, *In re Thorington,* 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) may be used to overcome an actual or provisional rejection based on a nonstatutory double patenting ground provided the conflicting application or patent is shown to be commonly owned with this application. See 37 CFR 1.130(b).

Effective January 1, 1994, a registered attorney or agent of record may sign a terminal disclaimer. A terminal disclaimer signed by the assignee must fully comply with 37 CFR 3.73(b).

Claims 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, and 11 are provisionally rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 53, 65, 68, 69, 70, 71, 72, and 79 of copending Application No. 10/451,210. Although the conflicting claims are not identical, they are not patentably distinct from each other because the pending application and application '210 are directed to an adenoviral nucleic acid comprising a nucleic acid sequence coding for YB-1, wherein the adenoviral nucleic acid is E1 deficient. In addition, US '210 claims using a pharmaceutical comprising a cyostatic agent with the adenovirus to treat a tumor disease (claim 71).

This is a provisional obviousness-type double patenting rejection because the conflicting claims have not in fact been patented.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Brian Whiteman whose telephone number is (571) 272-0764. The examiner can normally be reached on Monday through Friday from 7:00 to 4:00 (Eastern Standard Time), with alternating Fridays off.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, John LeGuyader, SPE - Art Unit 1635, can be reached at (571) 272-0760.

Papers related to this application may be submitted to Group 1600 by facsimile transmission. Papers should be faxed to Group 1600 via the PTO Fax Center located in Crystal Mall 1. The faxing of such papers must conform with the notice published in the Official Gazette, 1096 OG 30 (November 15, 1989). The CM1 Fax Center number is (703) 872-9306.

Any inquiry of a general nature or relating to the status of this application or proceeding should be directed to the receptionist whose telephone number is (703) 308-0196.

Brian Whiteman
Patent Examiner, Group 1635

JOHN L. LeGUYADER
SUPERVISORY PATENT EXAMINER
TECHNOLOGY CENTER 1600

INFORMATION DISCLOSURE CITATION

(Use several sheets if necessary)

ATTY DOCKET NO.	SERIAL NO.
3961.002	10/029,574
APPLICANT(S)	
Per Sonne HOLM, et al.	
FILING DATE	GROUP
12/20/01	1632

U.S. PATENT DOCUMENTS

*EXAMINER INITIAL		DOCUMENT NUMBER	DATE	NAME	CLASS	SUBCLASS	FILING DATE IF APPROPRIATE
PV		5,856,181	01/05/99	McCormick	C12N	5/10	

FOREIGN PATENT DOCUMENTS

		DOCUMENT NUMBER	DATE	COUNTRY	CLASS	SUBCLASS	TRANSLATION YES	TRANSLATION NO
PV		WO 94/18992	09/01/94	World	A61K	35/76	✓	

OTHER DOCUMENTS *(Including Author, Title, Date, Pertinent Pages, Etc.)*

PV			Kirn, D. (reprint), et al.; Adenovirus E1A mutants that selectively replicate in and cause enhanced destruction of cancel cells in vitro and in nude mouse-human tumor xenografts; Cancer Gene Therapy, (Nov.-Dec. 1998), Vol. 5, No. 6, Supp.[s], pp. 079-079
PV			Bargou, Ralf C., et al.; Nuclear localization and increased levels of transcription factor YB-1 in primary human breast cancers are associated with intrinsic MDR1 gene expression; Nature Medicine, Vol. 3, No. 4, 1997, pp. 4470450, XP002160622, ISSN: 1078-8956

EXAMINER	DATE CONSIDERED
[signature]	6/2/04

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

INFORMATION DISCLOSURE CITATION

(Use several sheets if necessary)

ATTY DOCKET NO.	SERIAL NO.
3961.002	10/029,574
APPLICANT(S) Per Sonne HOLM, et al.	
FILING DATE 12/20/01	GROUP 1632

U.S. PATENT DOCUMENTS

*EXAMINER INITIAL		DOCUMENT NUMBER	DATE	NAME	CLASS	SUBCLASS	FILING DATE IF APPROPRIATE

FOREIGN PATENT DOCUMENTS

		DOCUMENT NUMBER	DATE	COUNTRY	CLASS	SUBCLASS	TRANSLATION YES	TRANSLATION NO

OTHER DOCUMENTS *(Including Author, Title, Date, Pertinent Pages, Etc.)*

BW			Heise, et al.; ONYX-015, an E+B gene-attenuated adenovirus, causes tumor-specific cytolysis and antitumoral efficacy that can be augmented by standard chemo-therapeutic agents; Nature Medicine, Vol. 3, No. 6, June 1, 1997, pp. 639-645, XP0020953838, ISSN: 1078-8956

EXAMINER	DATE CONSIDERED
Brian Whiteley	6/2/04

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

Notice of References Cited	Application/Control No. 10/029,574	Applicant(s)/Patent Under Reexamination HOLM ET AL.	
	Examiner Brian Whiteman	Art Unit 1635	Page 1 of 2

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	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Anderson et al., Nature, Vol. 392, pp. 25-30, 1998.
	V	Verma, Nature, Vol. 389, pages 239-242, 1997.
	W	Vile et al., Gene Therapy, Vol. 7, pp. 2-8, 2000.
	X	Gomez-Navarro et al., European Journal of Cancer, Vol. 35, pp. 867-885, 1999.

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

Notice of References Cited	Application/Control No. 10/029,574	Applicant(s)/Patent Under Reexamination HOLM ET AL.	
	Examiner Brian Whiteman	Art Unit 1635	Page 2 of 2

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	U	McNeish et al., Gene Therapy, pp. 1-7, 2004.
	V	Green et al., Cancer Gene Therapy, 9:1036-1042 2002.
	W	Alemany et al., Nature Biotechnology, 18:723-727, 2000.
	X	Gromeier, ASM News, 68:438-445, 2002.

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

Human gene therapy

W. French Anderson

Although gene therapy as a treatment for disease holds great promise, progress in developing effective clinical protocols has been slow. The problem lies in the development of safe and efficient gene-delivery systems. This review will evaluate the problems and the potential solutions in this new field of medicine.

The first approved clinical protocol for somatic gene therapy started trials in September 1990[1]. Since then, in just 7½ years, more than 300 clinical protocols have been approved worldwide and over 3,000 patients have carried genetically engineered cells in their body. The conclusions from these trials are that gene therapy has the potential for treating a broad array of human diseases and that the procedure appears to carry a very low risk of adverse reactions; the efficiency of gene transfer and expression in human patients is, however, still disappointingly low. Except for anecdotal reports of individual patients being helped, there is still no conclusive evidence that a gene-therapy protocol has been successful in the treatment of a human disease. Why not?

In this review I will examine the 'why not?' by evaluating the promise and the problems of gene therapy. There are various categories of somatic cell gene therapy, distinguished by the mode of delivery of the gene to the affected tissue (see Box 1). The challenge is to develop gene therapy as an efficient and safe drug-delivery system. This goal is more difficult to achieve than many investigators had predicted 5 years ago. The human body has spent many thousands of years learning to protect itself from the onslaught of environmental hazards, including the incorporation of foreign DNA into its genome. Viruses, however, have been partially successful in overcoming these barriers and being able to insert their genetic material into human cells. Hence the initial efforts at gene therapy have been directed towards engineering viruses so that they could be used as vectors to carry therapeutic genes into patients. A number of reviews on aspects of gene therapy have been published recently[2–10]; this review will consider the categories of the various virus vectors in turn.

Vectors based on RNA viruses

Retroviruses were initially chosen as the most promising gene-transfer vehicles[11]. Currently, about 60% of all approved clinical protocols utilize retroviral vectors. These RNA viruses can carry out efficient gene transfer into many cell types and can stably integrate into the host cell genome (Fig. 1), thereby providing the possibility of long-term expression. They have minimal risk because retroviruses have evolved into relatively non-pathogenic parasites (although there are exceptions, such as the human immunodeficiency viruses (HIV) and human T-cell lymphotropic viruses (HTLV)). In particular, murine leukaemia virus (MuLV) has traditionally been used as the vector of choice for clinical gene-therapy protocols, and a variety of packaging systems to enclose the vector genome within viral particles have been developed. The vectors themselves have all of the viral genes removed, are fully replication-defective and can accept up to about 8 kilobases (kb) of exogenous DNA.

Box 1 The three categories of somatic cell gene therapy

- The first is *ex vivo*, where cells are removed from the body, incubated with a vector and the gene-engineered cells are returned to the body. This procedure is usually done with blood cells because they are the easiest to remove and return.
- The second is *in situ*, where the vector is placed directly into the affected tissues. Examples are the infusion of adenoviral vectors into the trachea and bronchi of patients with cystic fibrosis, the injection of a tumour mass with a vector carrying the gene for a cytokine or a toxin, or the injection of a vector carrying a dystrophin gene directly into the muscle of a patient with muscular dystrophy.
- The third is *in vivo*, where a vector could be injected directly into the blood stream. There are no clinical examples of this third category as yet, but if gene therapy is to fulfil its promise as a therapeutic option, *in vivo* injectable vectors must be developed.

The problems that investigators face in developing retroviral vectors that are effective in treating disease are of four main types: obtaining efficient delivery, transducing non-dividing cells, sustaining long-term gene expression, and developing a cost-effective way to manufacture the vector.

Obtaining efficient delivery. Clinical protocols with retroviral vectors primarily use the *ex vivo* approach. Currently, the cells that are transduced by retroviral vectors are those that possess a high level of the natural MuLV (amphotropic) receptor and are actively dividing at the time of exposure to the vector. Most human cells that can be grown *in vitro* can be transduced, although a few cell types cannot. An important target cell is the primitive haematopoietic stem cell (HSC) because gene transfer into these cells would result in gene-engineered cells for the life of the recipient. However, HSCs have a low level of amphotropic receptor and are poorly transducible[12]. The HSC remains, therefore, an important but elusive target.

The broad range of cell types possessing the amphotropic receptor, known to be a phosphate symport, limits the target-specific utility of these vectors in the *in vivo* approach. Using different viral envelope proteins that recognize different receptors (for example, the vesicular stomatitis virus (VSV)-G protein or the gibbon ape leukaemia virus (GALV) envelope protein) can vary the range of cells that can be transduced, but still does not provide much specificity. The difficulty is that, because retroviral vectors cannot be generated at a high titre (amphotropic vectors appear to be limited to 1×10^7 colony-forming units (CFU) per ml and VSV-G pseudotyped vectors to 1×10^9 CFU per ml), it is not possible to get a large number of vector particles to the desired cell type *in vivo*. The viral particles would bind to many cells they encounter and, therefore, would be diluted out before reaching their target (other issues, such as complement-mediated lysis, will be discussed later). The problem can be quantified. The human body contains about 5×10^{13} cells. If a 100 ml sample of retroviral vector were given to a patient, that would be about 1×10^9 active vector particles. Even if every vector particle were 100% efficient at infection, only 1 cell in 50,000 would be transduced. What is needed is a retroviral particle that will preferentially bind to its target cell and can be manufactured at a high titre.

Efforts to target specific cell types have centred on attempts to engineer the natural retroviral envelope protein. The envelope protein has two functions: binding to its receptor (by the surface (SU) moiety) and enabling the entry of the viral nucleoprotein core (carried out primarily by the transmembrane (TM) moiety). The SU protein binds to its receptor on the target cell surface and, as a result, the SU/TM complex undergoes a conformational change that allows fusion of the viral and cellular membranes, followed by entry of the viral core (which carries the virus's genetic information) into the target cell's cytoplasm (Fig. 1).

Two broad approaches to providing target cell specificity have been followed. First, the natural receptor-binding domain of the SU protein has been replaced with a ligand or single-chain antibody that recognizes a specific cell surface receptor[13,14]. A wide range of receptors have

been targeted, but the difficulty is that even though specific binding can be obtained between the engineered vector and the target cell receptor, gene transfer has been unacceptably low in all these experiments. The reason is clear. The retroviral envelope protein is thought to be a trimer with a complex quaternary structure[15]. When the natural receptor-binding domain is replaced by a foreign sequence, the whole structure of the envelope protein is altered. The result is that the natural post-binding conformational change that leads to the fusion of the virus with the cell membrane does not occur. Without fusion, core entry and gene transfer do not take place efficiently.

Engineering the receptor-binding domain of SU while maintaining the ability of the envelope protein to carry out core entry will require a better understanding of the structure–function relationships within the envelope protein complex. This understanding has been enhanced by the recent publication of the three-dimensional structure of the receptor-binding domain of the murine ecotropic (Friend strain) SU protein[16]. It should now be possible to engineer ligands into very specific sites in the SU protein with a higher probability of maintaining the functional properties of the envelope protein for core entry.

Other structure–function studies of the retroviral envelope protein are also contributing to our understanding of how to obtain efficient core entry after binding. The three-dimensional structure of a portion of the Moloney ecotropic retroviral TM protein was published last year[15]. Recently it has been shown that the separate monomers in the predicted trimeric structure of the envelope can cross-talk with each other[17]. In other words, separate monomers, each of which is defective, can complement each other to provide an active trimeric envelope. Using this technique it has been possible to define separate functional domains in the TM protein[18]. As the complete three-dimensional structure and functional domains of the envelope protein become known, constructing retroviral vectors that are able to target specific cells with high efficiency should be possible.

Progress has been made using a second broad approach to targeting that could be called 'tethering'. Although several creative systems have been designed[7], the most successful approach at present appears to be insertion of a ligand that recognizes an extracellular matrix (ECM) component into a part of the SU protein that does not disturb the natural receptor-binding domain. This tethering concentrates the vector in the ECM in the vicinity of the target cells. Receptor binding and core entry can then occur through the natural envelope-receptor mechanism. Two ligands that appear particularly useful for tethering are those specific for fibronectin[19] and for collagen[20]. Fibronectin is present in normal ECM and exposed collagen is present in areas of damage, for example after wound injury as in the cardiovascular system after angioplasty.

Transduction of non-dividing cells. Although the inability of MuLV-based retroviral vectors to transduce non-dividing cells is very useful in some situations, for example when a toxin gene is being inserted into dividing cancer cells and not into the normal non-dividing cells (see below under 'Clinical studies'), there are many situations where one would want to insert a therapeutic gene into normal non-dividing cells. Many potential target cells are not actively dividing *in vivo*; only certain blood cells (not the stem cell) and the cells lining the gastrointestinal tract are continually in division. Lentiviruses (such as HIV-1) are able to infect non-dividing cells, but vectors constructed from these viruses raise concerns over safety because of the possibility that recombination could produce a pathogenic virus. Attempts to transfer into murine retroviral vectors the specific signals from HIV that allow transduction of non-dividing cells have not been successful. Recently it has become possible to use just 22% of the HIV genome (which does not include any of the genes that cause pathology) in a retroviral vector[21,22]. The chances of recombination have been further reduced by the use of a non-HIV envelope protein. This hybrid system holds great promise for providing the option of transducing non-dividing cells *in vivo* in a safe manner. Another RNA viral system being developed is based on the human foamy virus[23]. These vectors are able to transduce a broad range of cell types, are not inactivated by human serum, and may be able to transduce some non-dividing, as well as dividing, cells.

Improving gene expression. Assuming that efficient gene transfer can be developed, the next issue is long-term, stable gene expression at an appropriate level[6]. This is perhaps the greatest shortcoming of present vectors. Although gene expression is being discussed here under retroviral vectors, the topic applies to gene transfer vectors of all types.

Several factors are involved in maintaining the stable expression of genes after their transfer. First, the regulatory sequences that control gene expression often do not remain active. There is a tendency for the cell to recognize foreign promoters (particularly viral promoters such as simian virus 40 (SV40) and cytomegalovirus (CMV)) and inactivate them (by methylation or other mechanisms). The role of lymphokines, cytokines and other growth factors in maintaining gene expression is also poorly understood. Second, even if the gene stays active within the cell, the cell often dies. The immune system is designed to recognize and eliminate foreign gene products and cells that produce a foreign protein. All viral genes are eliminated from retroviral vectors, and so immune recognition of viral proteins (except for those, such as capsid proteins, that are packaged into the viral particle itself) is not an issue (but see the discussion of adenoviral vectors below). Nonetheless, the immune system is still likely to recognize a new or modified protein produced by the therapeutic gene; a newly synthesized normal protein will appear abnormal to an immune system that has never been exposed to it.

Use of a cell's own *cis*-regulatory DNA sequences will probably provide more stable long-term gene expression than can be obtained with viral promoters, but identifying all the components of a gene's regulatory system can be difficult. As an extreme case, the regulatory sequences involved in the proper regulation of the haemoglobin (β-globin) genes are spread over nearly 100 kb. Because a retroviral vector can only accommodate 6–8 kb of sequence, regulatory sequences may need to be truncated to their minimal essential length before being incorporated into such vectors. Even when the natural regulatory elements are used, they may not function correctly without the proper signals and feedback mechanisms that normally operate in the appropriate cellular milieu. For example, the insulin enhancer/promoter still cannot direct regulated expression when delivered to fibroblasts. Again, this emphasizes the need to develop vectors that are capable of gene transfer to specific cell types.

There is steady progress on these fronts, but long-term, stable, appropriate-level gene expression *in vivo* in a range of cell types is still to be accomplished. Once these hurdles are cleared, the next goal will be to achieve gene expression that can be regulated. Many important target genes, such as that for insulin, are not expressed at the same level all the time, but respond to physiological signals within the body. The goal is to use regulatory sequences that respond to the body's own physiological signals (so that inserted therapeutic genes can function the way that normal endogenous genes do) or to drugs that can be used to control the level of gene activity. In some cases, only low levels of essentially unregulated expression may be beneficial (for example, in haemophilia or adenosine deaminase (ADA) deficiency), whereas in other cases tight regulation may be required (for example, for insulin production in diabetes).

Manufacturing the vector. Although consideration of how a pharmaceutical company would be able to manufacture millions of doses of a gene-therapy vector was irrelevant a decade ago, this has now become a real issue. Retroviral vectors are biological agents: they can only be made by living cells. Biological systems are not the easiest systems in which to carry out good manufacturing practice (GMP) and quality assurance/quality control (QA/QC) procedures mandated by the Food and Drug Administration (FDA), as manufacturers of vaccines have learned.

One of the major concerns with retroviral vectors is the possibility that a replication-competent retrovirus (RCR) could arise during the manufacturing process. Because retroviral vectors are produced in

packaging cells that contain a packaging-defective viral genome, and because retroviruses have a high propensity for recombination, this possibility is always present. Furthermore, as every mammalian cell contains endogenous retroviruses, additional viral sequences could be incorporated into the RCR, perhaps producing a pathogenic virus.

Another potential problem results from the ability of retroviral vectors to integrate randomly into host cell DNA. For example, a vector might insert itself into a tumour suppressor gene, thereby increasing the propensity of the cell to become cancerous. The only example of unintentional tumour production in a retroviral gene transfer experiment in large animals was published in 1992; three cases of lymphoma were reported among ten rhesus monkeys whose bone marrow had been destroyed by irradiation and who were then transplanted with haematopoietic stem cells that had been exposed to a large number of RCR as well as the experimental vector[24]. It was shown that the cancers resulted from integration of an RCR (not of the retroviral vector), were clonal events and developed only after a long period (6–7 months) of retroviraemia.

The subject of RCR production and safety as well as of potential tumour production was extensively analysed in a report to the NIH Recombinant DNA Advisory Committee (RAC) and the FDA[25]. The conclusion was that the current QA/QC procedures required by the FDA make it exceedingly unlikely that any patient could receive sufficient RCR to produce either a retroviraemia or a malignancy. However, the manufacturing and testing process to ensure this degree of safety is complex and expensive.

As the goal of present research is the production of a gene therapy vector that can be injected directly into the body (just like penicillin or insulin), additional problems must be considered. For example, mouse packaging cells produce retroviral vectors that are destroyed by human complement. Although this sensitivity makes the vector particles 'safer', it does markedly reduce their half-life *in vivo* and the efficiency of gene transfer. The major component of this sensitivity arises from the presence of unique sugar groups on viral glycoproteins produced in the murine packaging cells that make the viral particles sensitive to human complement. Either the vector particles produced in mouse cells must be engineered to avoid the human complement system, or the vector needs to be made in a non-murine packaging cell line that can provide the viral particles with appropriate sugar groups on their surface. However, as mentioned above, essentially all mammalian cells have their own endogenous retroviruses that could recombine with the vector to produce a new, potentially pathogenic, RCR; many of these endogenous viruses are still unknown. Although any cell line is suspect, the use of primate or human cells as packaging cells raises the greatest safety concerns in this regard. Human packaging cells can, however, be engineered to be very safe. For example, the ProPak cell line[26], which has the viral *gag–pol* genes on a separate DNA construct from the *env* gene (producing a 'split' packaging cell line) as well as other safety features, is certainly safer than the murine packaging cell line PA317, which is used for most of the present retroviral vector clinical trials.

These issues are resolvable, but it will take several years of product development to develop a cost-effective manufacturing system that will produce safe, efficient gene-therapy vectors on a sufficient scale to allow worldwide marketing. Although a non-viral delivery system that avoids many of these problems may be the gene-therapy vector of the future (see discussion below under 'Non-viral vectors'), the many present and future clinical protocols using retroviral vectors require that the manufacturing issues of safety and efficiency be solved.

Vectors based on DNA viruses

Adenoviral vectors. The DNA virus used most widely for *in situ* gene transfer vectors is the adenovirus (specifically serotypes 2 and 5). Adenoviral vectors have several positive attributes: they are large and can therefore potentially hold large DNA inserts (up to 35 kb, see below); they are human viruses and are able to transduce a large number of different human cell types at a very high efficiency (often reaching nearly 100% *in vitro*); they can transduce non-dividing cells; and they can be produced at very high titres in culture. They have been the vector of choice for several laboratories trying to treat the pulmonary complications of cystic fibrosis, as well as for a variety of protocols attempting to treat cancer.

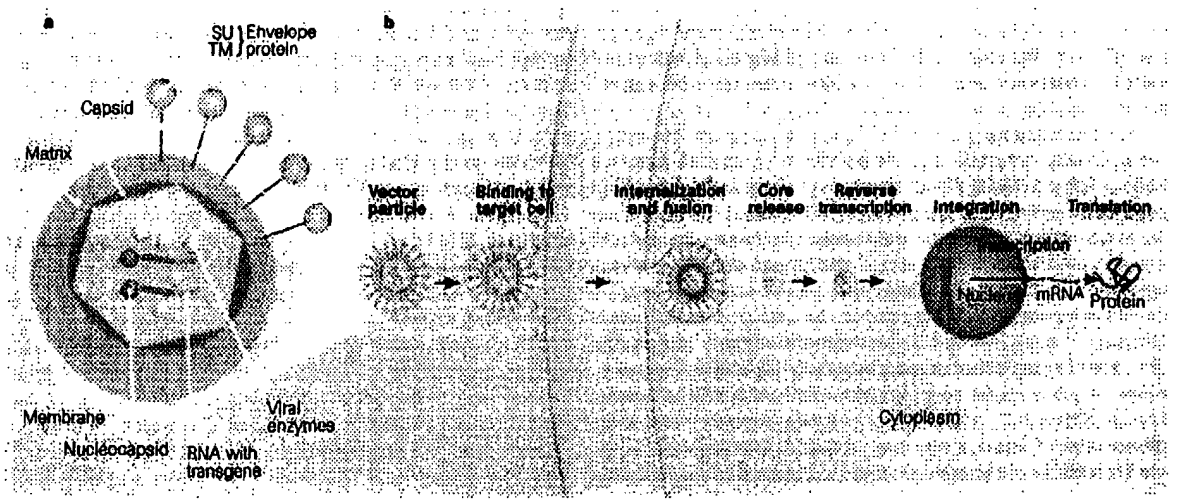

Figure 1 The protocol for retroviral vectors. **a**, Diagram of a retroviral vector. The vector particle is covered by a membrane (derived from the membrane of the cell from which the viral particle budded). Anchored in the membrane is the envelope protein, which is composed of two parts: the SU (surface) protein, which is responsible for binding to the receptor on the target cell, and the TM (transmembrane) protein, which passes through the membrane into the viral matrix and is involved in the fusion step. Beneath the viral membrane is the matrix protein and deeper still is the viral core, which is composed of a surrounding capsid within which are two identical strands of RNA together with the nucleocapsid protein and the viral enzymes (protease, polymerase and integrase). In a retroviral vector the viral genes have been replaced by a transgene. **b**, Diagram of a retroviral vector transducing a target cell. First, the vector particle binds to its receptor on the target cell by means of its SU envelope protein. The particle is then internalized into the cytoplasm of the cell, encased inside an endosome. The envelope protein initiates fusion of the viral membrane with the endosomal membrane, causing the viral core to be released into the cytoplasm. Reverse transcription takes place within the core, which results in the RNA being copied into a double strand of DNA. The double-stranded viral DNA then enters the nucleus, integrates into the chromosomal DNA, and is transcribed. Because the viral genes have been replaced by a transgene, only the protein product of the transgene is made instead of new viral particles.

Adenoviral vectors have certain drawbacks, however. First-generation vectors were deleted for the early region 1 (E1) functions in order to render them replication-defective. In addition, these vectors were deleted in the E3 region in order to create space for the insertion of transgenes. The E3 region, as discussed below, functions to suppress the host immune response during virus infection, but is not required for replication or packaging *in vitro*. Vectors with E1 and E3 deleted elicited strong inflammatory and immune responses[27]. This is thought to be a consequence of 'leaky' expression of adenoviral proteins in the transduced cells because these first-generation vectors retain most of the viral genome. It was hoped that a weaker immune response would result if additional viral genes were deleted. Thus vectors with the deletion of E1 coupled with the deletion of other essential early genes, E2a and/or E4 (refs 28, 29), or vectors with all of the viral genes deleted (so-called 'gutless' vectors[30-32]) have been constructed and tested in animals There have been conflicting reports regarding the immunogenicity, stability of gene expression, and persistence *in vivo* of gutless vectors[33]. In fact, these properties may differ depending on the exact vector design, the tissue type that the vector is introduced into, and the nature of the transgene insert. In particular, the gutted vectors offer the possibility of introducing up to 35 kb of genomic sequences, and it has been suggested that inclusion of nuclear matrix attachment regions might facilitate long-term gene expression and persistence of the vector sequences.

Deleting more and more viral genes may not always be advantageous because some of these genes may have beneficial attributes, for example suppressing an immune response against the vector. Their removal could increase the rate at which the vector is eliminated. As an example, the E3 region encodes a protein of relative molecular mass 19K that protects the virus, and presumably the engineered cells, from host immune surveillance[34]. Various effector mechanisms may be involved in viral vector clearance[35]. In addition, *cis*-acting sequences may exist that help maintain the stability of the adenoviral genome in the cell. As with drug trials, results in animals (even in primates) have not always reflected what happens in patients. Vectors that produce inflammatory responses in primates may not do so in human patients, and the opposite situation is probably also likely. Recently, the first 'true' phase I gene therapy clinical trials have begun: normal volunteers have been tested with intradermal injection (and now by intrabronchial infusion) of adenoviral vectors in order to determine the immunological response to adenoviral vectors in human beings.

By engineering the correct combination of viral genes (incorporating immunosuppressive genes, perhaps from various sources, while deleting immune-stimulating gene products and reducing, if possible, the immunogenicity of viral capsid proteins), it is likely that adenoviral vectors can be generated that have low toxicity, that do not generate an immune response, and that, therefore, can be given repeatedly. The latter point is important because adenoviral vectors do not integrate and they survive in the cell for a limited time (although in non-dividing cells this may be for an extended period). The ability to administer the vector repeatedly will be critical in many treatment protocols, for example in those for haemophilia and cystic fibrosis. Although it would clearly be optimal to engineer vectors that do not elicit an immune response, an interim solution could be to use transient immunosuppression of the patient to allow repeated administration of vectors. Another approach is to blockade costimulatory interactions required for an immune response to an antigen, thereby transiently 'blinding' the immune system during vector administration and making repeat administration possible.

Adeno-associated viral vectors. Another DNA virus used in clinical trials is the adeno-associated virus (AAV). This is a non-pathogenic virus that is widespread in the human population (about 80% of humans have antibodies directed against AAV). Initial interest in this virus arose because it is the only known mammalian virus that shows preferential integration into a specific region in the genome (into the short arm of human chromosome 19). As the virus does not produce disease, its insertion site appears to be a 'safe' region in the genome. It would be useful, therefore, to engineer the sequences that dictate this site-specific insertion into gene-therapy vectors. Unfortunately, the present AAV vectors appear to integrate in a nonspecific manner[36], although it has been suggested that vectors could be designed that retain some specificity[37].

Even though integration site specificity has not been achieved, AAV vectors have been shown to transduce brain, skeletal muscle, liver and possibly CD34$^+$ blood cells efficiently[2,38-40]. There are several drawbacks, however: some cells require a very high multiplicity of infection (the number of viral particles per cell required to achieve transduction); the AAV genome is small, only allowing room for about 4.8 kb of added DNA; and the production of viral particles is still very labour intensive because efficient packaging cells have not yet been developed. However, these vectors hold promise and appear to be safe. Furthermore, AAV may be capable of integrating into non-dividing cells, although again this desirable attribute of the wild-type virus appears to be lost from the vectors, which can enter non-dividing cells but remain in an episomal state until cell division occurs.

Other DNA virus-based vectors

Other DNA viruses are being studied as possible gene-therapy vectors for specific situations. For example, herpes simplex virus (HSV) vectors have a propensity for transducing cells of the nervous system[41,42], as well as several other cell types. A stripped-down version of the HSV, called an amplicon, may have certain advantages, particularly when combined with components from other viral systems[43]. A number of other DNA virus vectors are under study including poxviruses.

Several investigators are examining replication-competent, or attenuated, viral vectors (both DNA and RNA). In addition, hybrid systems have been reported where an adenoviral vector is used to carry a retroviral vector into a cell that is normally inaccessible to retroviral transduction[44].

Non-viral vectors

Although viral systems are potentially very efficient, two factors suggest that non-viral gene delivery systems will be the preferred choice in the future: safety, and ease of manufacturing. A totally synthetic gene-delivery system could be engineered to avoid the danger of producing recombinant virus or other toxic effects engendered by biologically active viral particles. Also, manufacturing a synthetic product should be less complex than using tissue culture cells as bioreactors, and QA/QC procedures should be simplified. The reader is referred to the review on non-viral vectors entitled 'Drug delivery and targeting' by Robert Langer on pages 5–10 of this issue.

Table 1 Disease targets and gene-therapy protocols

(a) Types of gene therapy clinical protocols*

Type	Number	Percentage of total
Therapy	200	(86%)
Marker	30	(13%)
Non-therapeutic†	2	(1%)
Total	232	(100%)

(b) Disease targets for therapeutic gene therapy clinical protocols

Target	Number	Percentage of total
Cancer	138	(69%)
Genetic diseases	33	(16.5%)
CF	16	
Other‡	17	
AIDS	23	(11.5%)
Other§	6	(3%)
Total	200	(100%)

* Roughly 60% of all protocols use retroviral vectors, 20% use non-viral delivery systems, 10% use adenoviral vectors and the remainder use other viral vectors.
† A 'non-therapeutic' protocol means a non-therapeutic portion of a non-gene therapy clinical protocol.
‡ These 17 include 12 other monogenic diseases.
§ The five 'other' are: peripheral artery disease, rheumatoid arthritis, arterial restenosis, carpal tunnel syndrome and coronary artery disease (2).

Clinical studies

At present over 300 clinical protocols have been approved. Detailed information is available on the 232 protocols that had been approved in the USA as of 3 February 1998[45] (Table 1).

Only one phase III and several phase II clinical trials are now underway; all the rest of the approved gene therapy clinical protocols are for smaller phase I/II trials. Genetic Therapy Inc./Novartis is carrying out the phase III clinical trial. The target disease is glioblastoma multiforma, a malignant brain tumour[46]. The rationale is to insert a gene capable of directing cell killing into the tumour while protecting the normal brain cells. The retroviral vector used (G1TkSvNa) contains the neomycin-resistance gene as a selective marker and the herpes simplex thymidine kinase (HSTk) gene. The actual material injected into the tumour mass is a mouse producer cell line (PA317) which generates retroviral particles carrying the G1TkSvNa vector. As the only dividing cells in the area of a growing brain tumour are the tumour cells and cells of the vasculature supplying blood to the tumour, and retroviral vectors only transduce dividing cells, the only cells to receive the vector should be the cells of the tumour and its blood vessels. The viral HSTk can add a phosphate group to a non-phosphorylated nucleoside, whereas the endogenous human thymidine kinase cannot. Therefore, when an abnormal nucleoside, such as the drug ganciclovir, is given to the patient, only the cells expressing the HSTk gene will phosphorylate the drug, incorporate it into their DNA synthesis machinery and be killed.

In the current phase III clinical trial, mouse producer cells making vector particles carrying the HSTk gene are inoculated into residual tumour and peritumour areas following tumour resection. After 7 days, the patient is treated with ganciclovir[47]. In theory, the tumour cells that have been transduced with the vector containing the HSTk gene will phosphorylate ganciclovir; the ganciclovir triphosphate then blocks the DNA synthesis machinery and kills the cells.

In fact, at least four distinct mechanisms contribute to tumour cell death in this protocol. First is the direct effect of phosphorylated ganciclovir on the transduced tumour cells; second is the 'bystander' effect in which toxic agents (ganciclovir triphosphate) pass into neighbouring cells through gap junctions and kill them; third, is the local inflammatory effect caused by the injected mouse cells; and fourth is a systemic immune response. The phase III trial includes a total of more than 40 centres in North America and Europe and is scheduled to enrol a total of 250 patients. By the end of December 1997 over 200 patients had been enrolled.

Several phase II trials are underway testing gene-therapy vectors as 'vaccines', either against cancer[48] or against AIDS[49]. Vical has two active phase II trials using a plasmid containing the gene for the HLA-B7/β_2-microglobulin protein formulated with cationic lipids. One trial is for metastatic malignant melanoma and the other for head and neck squamous cell carcinoma. The concept is that an HLA gene (such as B7) that the tumour does not express is injected into the tumour mass and that expression of this foreign antigen should stimulate the immune system to react against the cancer. The data so far suggest that the immune system not only develops a response against the B7 antigen but also to other antigens on the tumour cells, thereby resulting in an immune attack on non-transduced tumour cells[50]. Viagen/Chiron has completed a phase II trial of about 200 patients over 2 years in which a retroviral vector encoding the *env* and *rev* gene segments of the HIV-1(IIIB) strain is injected intramuscularly to induce augmented anti-HIV cytotoxic T-cell responses as a treatment for AIDS. Unfortunately, determination of the efficacy of this treatment was made impossible by the advent of triple drug therapy for HIV infection, but no evidence of toxicity was seen.

Finally, a comment on the original adenosine deaminase (ADA) deficiency gene-therapy trial[1,51]. ADA deficiency is a rare genetic disorder that produces severe immunodeficiency in children. Starting in 1990, gene-corrected autologous T lymphocytes were given to two girls suffering from this disease. Both girls are doing well and continue to lead essentially normal lives. Patient 1 (A.D.) received a total of 11 infusions, the last being in the summer of 1992. Her total T-cell level and her level of transduced T cells have remained essentially constant for the past 5½ years. She contracted chickenpox in late 1996 and experienced the same clinical course as would have been expected for any normal 10-year-old. Both she and patient 2 (C.C.) continue to receive polyethylene glycol (PEG)-ADA. Although both girls have gene-engineered T lymphocytes in their circulation after more than 7 years, no definitive conclusion can be drawn as to the relative roles of PEG-ADA and gene therapy in their excellent clinical course.

Ethical issues

Somatic cell gene therapy for the treatment of serious disease is now accepted as ethically appropriate. Indeed, it is so well accepted, and the side effects from gene-therapy protocols have been so minimal, that the danger now exists that genetic engineering may be used for non-disease conditions, that is for functional enhancement or 'cosmetic' purposes. The first Gene Therapy Policy Conference organized by the NIH RAC focused on this issue in September 1997. The conclusion was that enhancement engineering is about to take place, and could slip through the regulatory process if RAC and the FDA (and similar organizations in other countries) are not vigilant. As an example, a US biotechnology company has developed the technology for transferring genes (specifically the tyrosinase gene) into hair follicle cells[52]. They are now looking for genes that promote hair growth with the clinical objective of reversing the hair loss that occurs after chemotherapy in cancer patients. The application to the FDA for product licensing would list chemotherapy-induced alopecia as the product indication. The risk–benefit analysis here would be very favourable. However, once a product is licensed for any indication, it can be prescribed by physicians for any 'off-label' use that is felt by the physician to be clinically justified. The result could be millions of balding men receiving gene therapy to treat their hair loss. The conference concluded that the FDA should use a risk–benefit analysis that takes into account the extensive off-label usage for cosmetic reasons that could take place.

Using genetic engineering to treat baldness is not a major issue in itself, of course. But this is just one example of how our society is moving towards a slippery slope where genetic engineering might very well be used for a broad range of enhancement purposes, including larger size from a growth hormone gene, increased muscle mass from a dystrophin gene and so on. If we knew that there would be no long-term negative effects of genetic engineering, then widespread, or even frivolous, use of genetic engineering technology might not be detrimental. But just as with nuclear energy, pesticides and fluorocarbons, we as a society tend to see the benefits but are caught off guard by the bad effects of our powerful new technologies. What society wants to do 100 years from now with regards to genetic engineering is their business, but it is our duty to begin the era of genetic engineering in as responsible a manner as possible. Until we have learned about the long-term effects of somatic cell gene therapy in the treatment of disease, we should not use this technology for any other purpose than where it is medically indicated[53].

In utero somatic gene therapy of the fetus will be undertaken in the foreseeable future. The same care should be exercised here as with somatic cell gene-therapy protocols for adults, children and newborns. So long as only serious disease is targeted and the risk–benefit ratios for both mother and the fetus are acceptable, *in utero* gene therapy should be ethically appropriate[54]. Germline gene therapy should not be attempted at this time for the reasons outlined elsewhere[55].

A situation with the potential for real abuse of the new technologies would be the combination of cloning and genetic engineering. This combination has already been achieved in sheep where single cells have been obtained from fetal fibroblasts, transduced with a gene (human factor IX), and the gene-engineered cells grown into living sheep producing human factor IX[56]. Attempts to use such techniques to produce genetically engineered humans would provoke an even greater ethical storm than the present suggestion by a Chicago scientist to clone humans.

The future

The ultimate goal of gene-therapy research is the development of vectors that can be injected, will target specific cells, will result in safe and efficient gene transfer into a high percentage of those cells, will insert themselves into appropriate regions of the genome (or will persist as stable episomes), will be regulated either by administered agents or by the body's own physiological signals, will be cost-effective to manufacture and will cure disease. As the number of target cells may be in the billions, very high efficiency of gene transfer and the injection of a large number of gene-therapy vectors may be necessary. How soon can we expect significant progress in each of these areas?

The next 5 years should bring the first successes for gene therapy, that means statistically significant data that a gene-therapy protocol results in significant improvement in the clinical condition of patients. Within this time frame the first vectors that can target specific tissues should begin clinical trials and tissue-specific gene expression should have made its way into clinical trials.

In a time frame of 5–15 years from now, I expect that the number of gene-therapy products will begin to increase exponentially, coinciding with the enormous increase in characterized genes as a result of the Human Genome Project. The first injectable vectors will reach clinical trials and efficient tissue-specific gene transfer will be available in a few cases. It will probably take longer to develop site-specific integration, efficiently regulated genes and the correction of genes *in situ* by means of homologous recombination. Beyond this, our imagination is the limit.

For many gene-therapy applications in the future, it is probable that a synthetic hybrid system will be used that incorporates engineered viral components for target-specific binding and core entry, immunosuppressive genes from various viruses and some mechanism that allows site-specific integration, perhaps utilizing AAV sequences or an engineered retroviral integrase protein. In addition, regulatory sequences from the target cell itself will be utilized to allow physiological control of expression of the inserted genes. All these components would be assembled *in vitro* in a liposome-like formulation with additional measures taken to reduce immunogenicity such as concealment by PEG.

Conclusions

Gene therapy is a powerful new technology that still requires several years before it will make a noticeable impact on the treatment of disease. Several major deficiencies still exist including poor delivery systems, both viral and non-viral, and poor gene expression after genes are delivered. The reason for the low efficiency of gene transfer and expression in human patients is that we still lack a basic understanding of how vectors should be constructed, what regulatory sequences are appropriate for which cell types, how *in vivo* immune defences can be overcome, and how to manufacture efficiently the vectors that we do make. It is not surprising that we have not yet had notable clinical successes. Nonetheless, the lessons we are learning in the clinic are invaluable in illuminating the problems that future research must solve.

Despite our present lack of knowledge, gene therapy will almost certainly revolutionize the practice of medicine over the next 25 years. In every field of medicine, the ability to give the patient therapeutic genes offers extraordinary opportunities to treat, cure and ultimately prevent a vast range of diseases that now plague mankind. □

W. French Anderson is at the Gene Therapy Laboratories, University of Southern California School of Medicine, Norris Cancer Center, 1441 Eastlake Avenue, Los Angeles, California 90033-0800, USA.

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Gene therapy — promises, problems and prospects

Inder M. Verma and Nikunj Somia

In principle, gene therapy is simple: putting corrective genetic material into cells alleviates the symptoms of disease. In practice, considerable obstacles have emerged. But, thanks to better delivery systems, there is hope that the technique will succeed.

In 1990, the first clinical trials for gene-therapy approaches to combat disease were carried out. Conceptually, the technique involves identifying appropriate DNA sequences and cell types, then developing suitable ways in which to get enough of the DNA into these cells. With efficient delivery, the therapeutic prospects range from tackling genetic diseases and slowing the progression of tumours, to fighting viral infections and stopping neurodegenerative diseases. But the problems — such as the lack of efficient delivery systems, lack of sustained expression, and host immune reactions — remain formidable challenges.

Although more than 200 clinical trials are currently underway worldwide, with hundreds of patients enrolled, there is still no single outcome that we can point to as a success story. To explore why this is the case, we will use our own experience and other examples to look at the many technical, logistical and, in some cases, conceptual hurdles that need to be overcome before gene therapy becomes routine practice in medicine.

At present, gene therapy is being contemplated only on somatic (essentially, non-reproductive) cells. Although many somatic tissues can receive therapeutic DNA, the choice of cell usually depends on the nature of the disease. Sometimes a clear definition of the target cell is needed. For example, the gene that is defective in cystic fibrosis has been identified, and clinical trials to deliver DNA as an aerosol into the lung have already begun[1]. Although cystic fibrosis is manifest in this organ, it is still not clear that delivery of a correcting gene by this method will reach the right type of cell. On the other hand, to correct blood-clotting disorders such as haemophilia, all that is needed is a therapeutic level of clotting protein in the plasma[2]. This protein may be supplied by muscle or liver cells, fibroblasts, or even blood cells[3–5]. The choice of tissue in which to express the therapeutic protein will also ultimately depend on considerations such as the efficiency of gene delivery, protein modifications, immunological status, accessibility and economics.

We also need to consider how much of the therapeutic protein should be delivered. In haemophilia B, which is caused by a deficiency of a blood-clotting protein called factor IX, giving patients just 5% of the normal circulating levels of this protein can substantially improve their quality of life[2]. Most people have about 5 μg of factor IX per millilitre of plasma, produced by the 10^{13} cells that make up the liver. So we need to deliver a correcting gene to 5×10^{11} cells — that is, 5% of liver cells. Alternatively, fewer liver cells would need to be modified if more factor IX could be produced per cell, without being deleterious. In the brain, however, gene transfer to just a few hundred cells could considerably benefit patients with neurological disease. And finally, we can consider the transfer of genes to a handful of stem (or progenitor) cells, which grow and divide to generate millions of progeny. The range in the number of cells that this technology has to cover is vast.

The Achilles heel of gene therapy is gene delivery, and this is the aspect that we will concentrate on here. Thus far, the problem has been an inability to deliver genes efficiently and to obtain sustained expression. There are two categories of delivery vehicle ('vector'). The first comprises the non-viral vectors, ranging from direct injection of DNA to mixing the DNA with polylysine or cationic lipids that allow the gene to cross the cell membrane. Most of these approaches suffer from poor efficiency of delivery and transient expression of the gene[6]. Although there are reagents that increase the efficiency of delivery, transient expression of the transgene is a conceptual hurdle that needs to be addressed.

Most of the current gene-therapy approaches make use of the second category — viral vectors. Importantly, the viruses used have all been disabled of any pathogenic effects. The use of viruses is a powerful technique, because many of them have evolved a specific machinery to deliver DNA to cells. However, humans have an immune system to fight off the virus, and our attempts to deliver genes in viral vectors have been confronted by these host responses. ▶

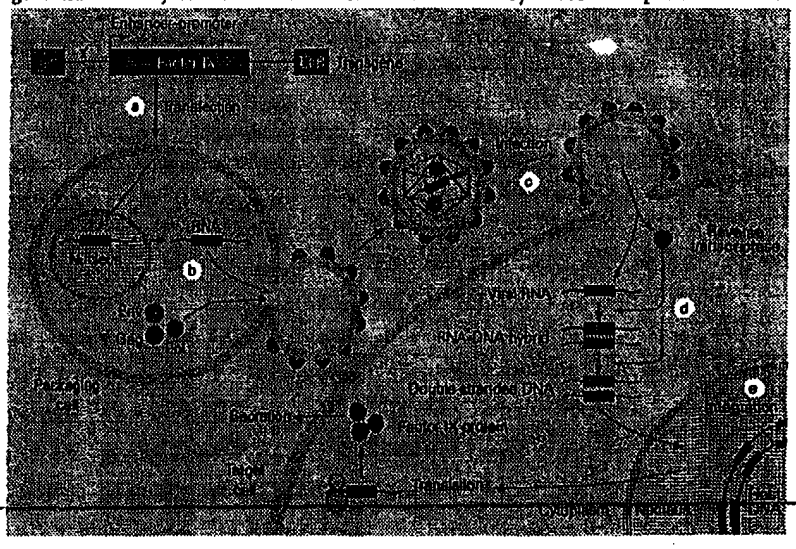


Figure 1 To create the retroviral vectors that are used in gene therapy, the life-cycles of their naturally occurring counterparts are exploited. a, The transgene (in this case, the gene for factor IX) in a vector backbone is put into a packaging cell, which expresses the genes that are required for viral integration (*gag*, *pol* and *env*). b, The transgene is incorporated into the nucleus, where it is transcribed to make vector RNA. This is then packaged into the retroviral vector, which is shed from the packaging cell. c, The vector is delivered to the target cell by infection. The membrane of the viral vector fuses with the target cell, allowing the vector RNA to enter. d, The virally encoded enzyme reverse transcriptase converts the vector RNA into an RNA–DNA hybrid, and then into double-stranded DNA. e, The vector DNA is integrated into the host genome, then the host-cell machinery will transcribe and translate it to make RNA and, in this case, factor IX protein. LTR, long terminal repeat; ψ, packaging sequence.

Retroviral vectors

Retroviruses are a group of viruses whose RNA genome is converted to DNA in the infected cell. The genome comprises three genes termed *gag*, *pol* and *env*, which are flanked by elements called long terminal repeats (LTRs). These are required for integration into the host genome, and they define the beginning and end of the viral genome. The LTRs also serve as enhancer–promoter sequences — that is, they control expression of the viral genes. The final element of the genome, called the packaging sequence (ψ), allows the viral RNA to be distinguished from other RNAs in the cell (Fig. 1)[7].

By manipulating the viral genome, viral genes can be replaced with transgenes — such as the gene for factor IX (Table 1). Transcription of the transgene may be under the control of viral LTRs or, alternatively, enhancer–promoter elements can be engineered in with the transgene. The chimaeric genome is then introduced into a packaging cell, which produces all of the viral proteins (such as the products of the *gag*, *pol* and *env* genes), but these have been separated from the LTRs and the packaging sequence. So, only the chimaeric viral genomes are assembled to generate a retroviral vector. The culture medium in which these packaging cells have been grown is then applied to the target cells, resulting in transfer of the transgene. Typically, a million target cells on a culture dish can be infected with one millilitre of the viral soup.

A critical limitation of retroviral vectors is their inability to infect non-dividing cells[8], such as those that make up muscle, brain, lung and liver tissue. So, when possible, the cells from the target tissue are removed, grown *in vitro*, and infected with the recombinant retroviral vector. The target cells producing the foreign protein are then transplanted back into the animal. This procedure has been termed '*ex vivo* gene therapy' and our group has used it to infect mouse primary fibroblasts or myoblasts (connective-tissue and muscle precursors, respectively) with retroviral vectors producing the factor IX protein. But within five to seven days of transplanting the infected cells back into mice, expression of factor IX is shut off[3,5,9]. This transcriptional shut-off has even been observed in mice lacking a functional immune system (nude mice), and it cannot be due to cell loss or gene deletion[5] because the transplanted cells can be recovered.

What is the mechanism of this unexpected but intriguing problem? We do not yet know, but the exceptions may provide some clues. To obtain sustained expression in mouse muscle following the transplantation of infected myoblasts, we used the muscle creatine kinase enhancer–promoter to control transcription of the transgene. Unfortunately, this is a weak promoter, and only low levels of protein were produced. So, we generated a chimaeric vector in which the muscle creatine kinase enhancer was linked to a strong promoter from cytomegalovirus. Using this vector, sustained and high levels of factor IX were expressed when the infected myoblasts were transplanted back into mice. Remarkably, these expression levels remained unchanged for more than two years (the life of the animal). So we can override the 'off switch' and achieve higher levels of expression by using an appropriate enhancer–promoter combination. But the search for such combinations is a case of trial and error for a given type of cell.

Another formidable challenge to the *ex vivo* approach is the efficiency of transplantation of the infected cells. Attempts to repeat the long-term myoblast transplantation in haemophiliac dogs led to only short-term expression, because the infected dog myoblasts could not fuse with the muscle fibres. So perhaps successful animal models will prove inadequate when the same protocols are extended to humans. Moreover, these models are based on inbred animals — the outbred human population, with individual variation, will add yet another degree of complexity. The haematopoietic (blood-producing) system may offer an advantage for *ex vivo* gene therapy because resting stem cells can be stimulated to divide *in vitro* using growth factors and the transplantation technology is well established. But there is still a lack of good enhancer–promoter combinations that allow sustained production of high levels of protein in these cells.

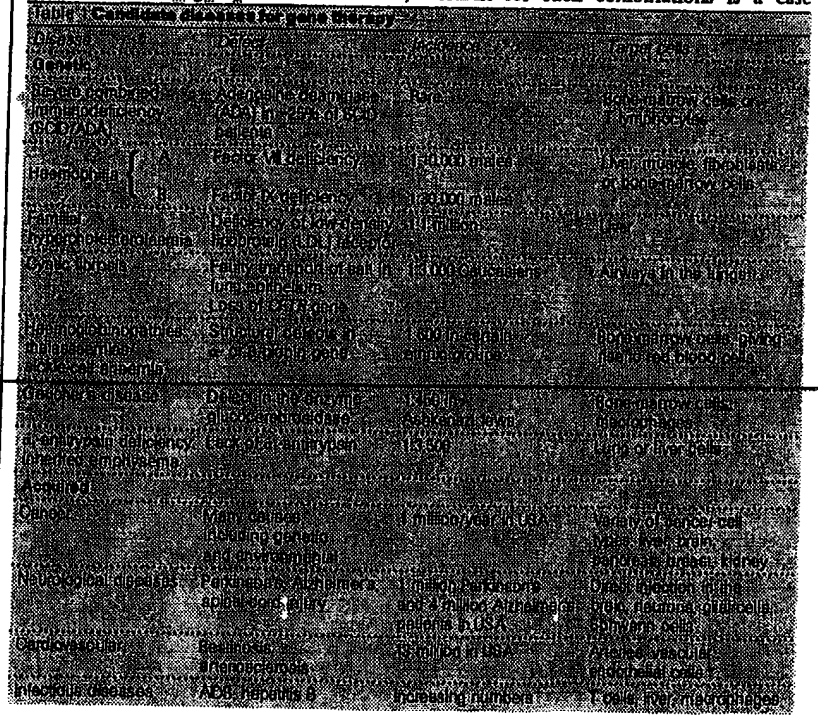
Another problem is the possibility of random integration of vector DNA into the host chromosome. This could lead to activation of oncogenes or inactivation of tumour-suppressor genes. Although the theoretical probability of such an event is quite low, it is of some concern (see section on clinical trials).

Lentiviral vectors

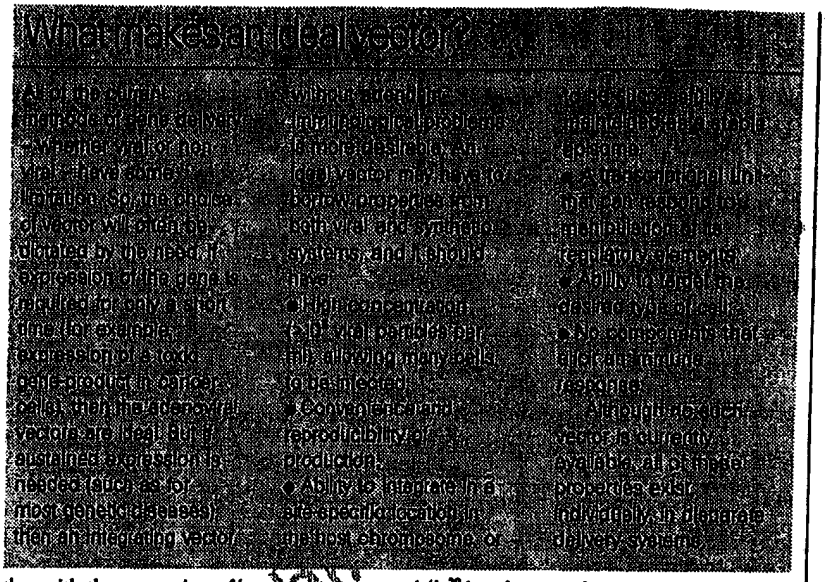
Lentiviruses also belong to the retrovirus family, but they can infect both dividing and non-dividing cells[10]. The best-known lentivirus is the human immunodeficiency virus (HIV), which has been disabled and developed as a vector for *in vivo* gene delivery. Like the simple retroviruses, HIV has the three *gag*, *pol* and *env* genes, but it also carries genes for six accessory proteins termed *tat*, *rev*, *vpr*, *vpu*, *nef* and *vif*[11].

Using the retrovirus vectors as a model, lentivirus vectors have been made, with the transgene enclosed between the LTRs and a packaging sequence[12]. Some of the accessory proteins can be eliminated without affecting production of the vector or efficiency of infection. The *env* gene product would restrict HIV-based vectors to infecting only cells that express a protein called $CD4^+$ so, in the vectors, this gene is substituted with *env* sequences from other RNA viruses that have a broader infection spectrum (such as glycoprotein from the vesicular stomatitis virus). These vectors can be produced — albeit on a small scale at the moment — at concentrations of $>10^9$ virus particles per ml (ref. 12).

When lentivirus vectors are injected into rodent brain, liver, muscle, eye or pancreatic-islet cells, they give sustained expression for over six months — the longest time tested so far[13,14]. Unlike the prototypical retroviral vectors, the expression is not subject to 'shut off'. Little is known about the possible immune problems associated with lentiviral vectors, but injection of 10^7 infectious units



does not elicit the cellular immune response at the site of injection. Furthermore, there seems to be no potent antibody response. So, at present, lentiviral vectors seem to offer an excellent opportunity for *in vivo* gene delivery with sustained expression.



Adenoviral vectors

The adenoviruses are a family of DNA viruses that can infect both dividing and non-dividing cells, causing benign respiratory-tract infections in humans[11]. Their genomes contain over a dozen genes, and they do not usually integrate into the host DNA. Instead, they are replicated as episomal (extrachromosomal) elements in the nucleus of the host cell. Replication-deficient adenovirus vectors can be generated by replacing the *E1* gene — which is essential for viral replication — with the gene of interest (for example, that for factor IX) and an enhancer–promoter element. The recombinant vectors are then replicated in cells that express the products of the *E1* gene, and they can be generated in very high concentrations ($>10^{11}$–10^{12} adenovirus particles per ml)[15].

Cells infected with recombinant adenovirus can express the therapeutic gene but, because essential genes for viral replication are deleted, the vector should not replicate. These vectors can infect cells *in vivo*, causing them to express very high levels of the transgene. Unfortunately, this expression lasts for only a short time (5–10 days post-infection). In contrast to the retroviral vectors, long-term expression can be achieved if the recombinant adenoviral vectors are introduced into nude mice, or into mice that are given both the adenoviral vector and immunosuppressing agents[16]. This indicates that the immune system is behind the short-term expression that is usually obtained from adenoviral vectors.

The immune reaction is potent, eliciting both the cell-killing 'cellular' response and the antibody-producing 'humoral' response. In the cellular response, virally infected cells are killed by cytotoxic T lymphocytes[16,17]. The humoral response results in the generation of antibodies to adenoviral proteins, and it will prevent any subsequent infection if the animal is given a second injection of the recombinant adenovirus. Unfortunately for gene therapy, most of the human population will probably have antibodies to adenovirus from previous infection with the naturally occurring virus.

It is possible that the target cell contains factors that might trigger the synthesis of adenoviral proteins, leading to an immune response. To try to get around this problem, second-generation adenoviral vectors were developed, in which additional genes that are implicated in viral replication were deleted. These vectors showed longer-term expression, but a decline after 20–40 days was still apparent[18]. This idea has now been taken further with the generation of 'gut-less' vectors — all of the viral genes are deleted, leaving only the elements that define the beginning and the end of the genome, and the viral packaging sequence. The transgenes carried by these viruses were expressed for 84 days[19].

There are considerable immunological problems to be overcome before adenoviral vectors can be used to deliver genes and produce sustained expression. The incoming adenoviral proteins that package DNA can be transported to the cytoplasm where they are processed and presented on the cell surface, tagging the cell as infected for destruction by cytotoxic T cells. So adenoviral vectors are extremely useful if expression of the transgene is required for short periods of time. One promising approach is to deliver large numbers of adenoviral vectors containing genes for enzymes that can activate cell killing, or immunomodulatory genes, to cancer cells. In this case, the cellular immune response against the viral proteins will augment tumour killing. Finally, although immunosuppressive drugs can extend the duration of expression, our goal should be to manipulate the vector and not the patient.

Adeno-associated viral vectors

A relative newcomer to the field, adeno-associated virus (AAV) is a simple, non-pathogenic, single-stranded DNA virus. Its two genes (*cap* and *rep*) are sandwiched between inverted terminal repeats that define the beginning and the end of the virus, and contain the packaging sequence[20]. The *cap* gene encodes viral capsid (coat) proteins, and the *rep* gene product is involved in viral replication and integration. AAV needs additional genes to replicate, and these are provided by a helper virus (usually adenovirus or herpes simplex virus).

The virus can infect a variety of cell types, and — in the presence of the *rep* gene product — the viral DNA can integrate preferentially[20] into human chromosome 19. To produce an AAV vector, the *rep* and *cap* genes are replaced with a transgene. Up to 10^{11}–10^{12} viral particles can be produced per ml, but only one in 100–1,000 particles is infectious. Moreover, preparation of the vector is laborious and, due to the toxic nature of the *rep* gene product and some of the adenoviral helper proteins, we currently have no packaging cells in which all of the proteins can be stably provided. Vector preparations must also be carefully separated from any contaminating adenovirus, and AAV vectors can accommodate only 3.5–4.0 kilobases of foreign DNA — this will exclude larger genes. Finally, we need more information about the immunogenicity of the viral proteins, especially given that 80% of the adult population have circulating antibodies to AAV. These considerations notwithstanding, AAV vectors containing human factor IX complementary DNA have been used to infect liver and muscle cells in immunocompetent mice. The mice produced therapeutic amounts of factor IX protein in their blood for over six months[21,22], confirming the promise of AAV as an *in vivo* gene-therapy vector.

Other vectors

Among the other viruses being considered and developed, is herpes simplex virus, which infects cells of the nervous system[23]. The virus contains more than 80 genes, one of which (*IE3*) can be replaced to create the vector. Around 10^8–10^9 viral particles are produced per ml, but the residual proteins are toxic to the target cell. Additional genes can be deleted, allowing more than one transgene to be included. But if essentially all of the viral proteins are deleted (gut-less vectors), only around 10^6 viral particles are produced per ml. And, again, many people have an immunity to components of herpes simplex virus, having already been infected at some time.

Vaccinia-virus-based vectors have also

been explored, largely for generating vaccines[24]. The Sindbis and Semliki Forest virus is being exploited as a possible cytoplasmic vector[25] which does not integrate into the nucleus. Although most of these systems produce the foreign protein only transiently, they add diversity to the available systems of gene transfer (Table 2).

Clinical trials

Over half of the 200 clinical trials that have been launched in the United States involve therapeutic approaches to cancer. Nearly 30 of them involve correction of monogenic diseases (Table 1) such as cystic fibrosis, α_1-antitrypsin deficiency and severe combined immunodeficiency (SCID). Most of the trials are phase I (safety) studies and, by and large, the existing delivery systems have no major toxicity problems. Moreover, lessons can be learned from previous clinical trials[26,27]. For example, seven years ago two patients were enrolled in a trial to correct deficiencies in adenosine deaminase (ADA, which leads to severe immunodeficiency). One of the patients improved, and makes 25% of normal ADA in her T cells, five years after the last infusion of infected T cells (although she is still treated with PEG–ADA; bovine adenosine deaminase mixed with polyethylene glycol). But in the other patient, the infected T cells could not produce enough of the deficient enzyme.

The efficacy of gene therapy cannot be evaluated until patients are completely taken off alternative treatments (in the above example, PEG–ADA). In another trial[28], weaning a patient away from PEG–ADA reduced the ability of the T cells to respond *in vitro* to a challenge by pathogens. Clearly, in these cases the retroviral vectors were not optimal, and the number of infected blood-progenitor cells was extremely low. However, these trials did help to establish the technology for generating clinical-grade recombinant retroviral particles, the procedures for infection and transplantation, and the protocols for monitoring patients and analysing data. The disappointing outcome probably lies in the inefficient gene-delivery system. We need a system in which the vector — containing the ADA gene — is efficiently delivered to progenitor cells, leading to sustained expression of high levels of the ADA protein. But, encouragingly, despite being repeatedly injected with highly concentrated recombinant viruses, the patients have suffered no untoward effects to date.

Future prospects

We now need a renewed emphasis on the basic science behind gene therapy — particularly the three intertwined fields of vectors, immunology and cell biology.

All of the available viral vectors arose from understanding the basic biology of the structure and replication of viruses. Clearly, existing vectors need to be streamlined further (see box on page 241), and vectors that target specific types of cell are being developed. The use of antibody fragments, ligands to cell-specific receptors, or chemical modifications to the vector need to be explored systematically. And advances such as the report — published only last week[29] — of the three-dimensional structure of the Env protein from mouse leukaemia virus (a commonly used vector), will facilitate the design of targeted vectors. A better understanding of gene transcription will allow us to design regulatory elements that permit promoter activity to be modulated, and development of tissue-specific enhancer–promoter elements should be vigorously pursued. Finally, we need to begin merging some of the qualities of viral vectors with non-viral vectors, to generate a safe and efficient gene-delivery system.

With respect to immunology, viruses still have many secrets to be unravelled. Viral systems that have evolved to escape immune surveillance can be incorporated into viral vectors. Some of these are being characterized; for example, the adenoviral E3 protein, the herpes simplex ICP47 protein and the cytomegalovirus US11 protein[30]. Systems from other pathogens may also be borrowed and incorporated into vectors. In some cases, the correcting protein will be sensed as foreign, eliciting an immune response. Animal models should help us to understand this and, where necessary, to develop strategies for tolerance.

Cell biology is involved because, in many cases, the goal of gene therapy is to correct differentiated cells, such as epithelial cells in cystic fibrosis and lymphoid cells in ADA deficiency. However, because these cells are continuously replaced there has to be either continued therapy or an attempt to target the stem cells. We first need to develop further the technologies for identifying and isolating these cells, to understand their regulation, and to target infection to them *in vivo*.

So how far have we come since clinical trials began? The promises are still great, and the problems have been identified (and they are surmountable). But what of the prospects? Our view is that, in the not too distant future, gene therapy will become as routine a practice as heart transplants are today. □

Inder M. Verma and Nikunj Somia are in the Laboratory of Genetics, The Salk Institute for Biological Studies, La Jolla, California 92037, USA.

Table 2 Comparison of properties of various vector systems

Features	Retroviral	Lentiviral	Adenoviral	AAV	Naked DNA
Magnitude of insert size	7–7.5 kb	7–7.5 kb	30 kb	4.5–4.9 kb	Unlimited size
Gene transfer (viral particles per ml)	10^6	10^6	10^{11}	10^6	Non-infectious
Route of gene delivery	Ex vivo	Ex vivo	Ex vivo	Ex vivo	Ex vivo
Integration	Yes	Yes	No	Yes/No	Very poor
Duration of expression in vivo	Long	Long	Short	Long	Short
Stability	Good	Not tested	Good	Good	Very good
Ease of preparation (scale up)	Difficult to scale up (titre 10–20 fold)	Not known	Easy to scale up	Difficult to purify, difficult to scale up	Easy to scale up
Immunological problems	Few	Few	Major problems	Not known	None
Pre-existing host immunity	Unlikely	Unlikely except SIV	Yes, maybe ADS+ patients	Yes	No
Safety problems	Insertional mutagenesis?	Insertional mutagenesis?	Inflammatory response, toxicity	Inflammatory response, toxicity	None

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MILLENNIUM REVIEW

Cancer gene therapy: hard lessons and new courses

RG Vile[1], SJ Russell[1] and NR Lemoine[2]

[1]*Molecular Medicine Program, Guggenheim 18, Mayo Clinic, 200 First Street SW, Rochester, Minnesota 55905, USA; and* [2]*ICRF Molecular Oncology Unit, Department of Cancer Medicine, Imperial College School of Medicine, London, UK*

Gene therapy for the treatment of cancer was initiated with high levels of optimism and enthusiasm. Recently, this perception has had to be tempered by the realisation that efficiency and accuracy of gene delivery remain the most significant barriers to its success. So far, there has been a disappointing inability to reach target cells with sufficient efficacy to generate high enough levels of direct killing and this has necessitated the invocation of bystander effects in order for any potential strategy to be convincing. At least in the foreseeable future, clinical advance will come from co-operation with other more established disciplines – such as chemotherapy, radiotherapy and immunotherapy. This is inevitable – and necessary – in order to prove that gene therapy can have efficacy as part of a combinatorial therapy, before hoping to move clinical mountains alone. In addition, there will have to be a thorough understanding of the clinical situations in which gene therapy will be used in order both to understand its own limitations, and to exploit its full potential. This will enable it to find the appropriate clinical niche in which its abilities will be optimally useful. Finally, anyone wishing to practise clinical cancer gene therapy will rapidly have to learn the ways of the free market and be able to juggle commercial necessities with ideological purity. Gene Therapy (2000) 7, 2–8.

Keywords: *cancer; gene therapy; immunotherapy; viral vector; targeting*

Introduction

The field of gene therapy for cancer now has the luxury (or some may even say the curse) of clinical trial data to assess the efficacy of both the genes and the vectors which have dominated the field through its infancy.[1–6] On balance, these data suggest that the problems which gene therapy for cancer will take into the new millennium focus far less on the choice of the therapeutic gene(s) to be used than on the means of delivering them. There is already a battery of genes that we know are very effective in killing cells, if they can but be expressed at the right site and at appropriate levels. None the less, until the perfectly targeted vector is developed,[7] the choice of gene will remain crucially important in order to compensate for the deficiencies of the vectors which we currently have available (Figure 1).[8]

Genes

There is still no clear consensus on which tumour-clearing approach should be adopted using gene transfer. Intuitively, it would seem that simple eradication of cells is likely to be the best, and safest. The self-renewing nature of malignant disease dictates that tumour cells should be cleared as efficiently as possible rather than genetically corrected. Thus, the most frequently used genes have been those designed to kill cells directly – such as suicide genes[9] – or to induce immune-mediated destruction – such as immunogenic antigens or cytokines.[10]

Correspondence: R Vile

None the less, several groups have had success using genetic therapies to turn the aberrant biology of tumour cells against themselves. This has been the basis for the design of replicating vectors targeting genetic mutations specific to tumour cells (see below),[11] as well as for the delivery of genes which stand as sentinels over control of the cell cycle such as p53.[5] Given the genetic redundancy in human tumours, especially at late stages of evolution, genetic therapies for cancer may run the risk of being short-circuited by the recruitment of other signals driving proliferation of the tumour cells. However, if the genetic targeting approach uses pathways which are central to the continued survival of the cell it may be possible to induce tumour killing by triggering apoptotic effector mechanisms directly[12,13] or indirectly.[14] In some cases, it is evident that tumour cells may actually have a particular vulnerability to the induction of programmed cell death because of the loss of survival signals, for instance through changes in their cell adhesion properties.[15] Whatever its mechanisms, no single gene can be a serious contender unless it has a demonstrable bystander effect. The requirement for such a bystander effect stems directly from the poor delivery efficiency provided by current vectors (see below).[7] This bystander effect can come in one of two guises – either local[9] or immune mediated[16,17] – and a combination of the two is preferable. The prototypical bystander killing involves the transfer of toxic metabolites locally between cells[9] but there may be other mechanisms including suppression of angiogenesis[18,19] and a 'kiss of death' delivered by contact with dying cells.[20] The next few years should see a wave of papers describing ways in which this local bystander killing can be enhanced, for example, by co-administration of pharmaceutical or genetic agents which

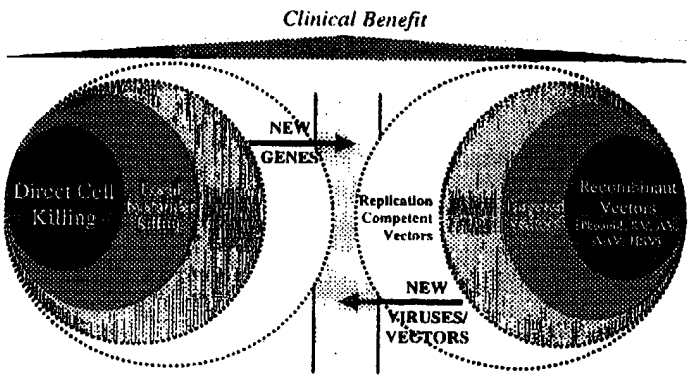

Figure 1 *The clinical efficacy of gene therapy is determined by the contribution of the potency of the therapeutic gene(s) used and the efficiency of delivery of those genes to tumour cells. Clinical benefit will result when the summation of the gene potency and the delivery efficiency combine to be able to reach and kill all of the tumour cells – either directly or indirectly through immune activation. The factors most likely to close the gap between the efficacy provided by gene potency or vector efficiency alone are the use of replicating vectors, more potent bystander effects and the effective stimulation of anti-tumour immune responses.*

enhance cell–cell communications.[21] In addition, immune stimulation through cell killing can also enhance local tumour killing[22] and can help to generate systemic immunity to other tumour deposits.[23] These effects can be active either over a short period of time through activation of non-specific immune effector mechanisms[22,24] or can set the foundations of long-lived, T cell-mediated immunity to tumour cells.[17,25] The most effective way to stimulate T cell-mediated immunity to tumours is to kill cells in such a way as to convince the immune system that they are the source of an aggressive, infection-like situation.[26] This mimics a pathological challenge against which the immune system is highly evolved to react. This contrasts with the scenario in which the chronic growth of tumour cells in a patient has, more likely than not, managed to 'tolerise' the immune system to the very cells which we would like it to attack.[27] Thus, exploitation of mechanisms that combine more powerful direct local killing with local and systemic immune activation will be a major priority. As well as the isolation of more potent versions of pre-existing genes such as HSVtk,[28] significant advances will come from the discovery of new genes which both kill more cells locally and which do so with as much immunological aggression ('Danger') as possible (Figure 1).[23,26]

Vectors

If the jury is still out on the choice of therapeutic gene, there seems to have been a much more definitive selection of vector type over the past few years. To date, cancer gene therapy trials have variously used the three most common vectors (plasmid, retrovirus and adenovirus). However, except for the situation where tumour/immune cells are manipulated *ex vivo*, there will be a clear preference in the coming years for the use of adenoviral vectors for *in vivo* delivery to tumours.[29] Dominant in this selection process is the high titre of adenoviruses ($>10^{11}$ p.f.u./ml) compared with other vectors. Given the requirement to kill, rather than correct, target cells, there is generally little need for integration (ie long-term gene expression as provided by C-type and lentiviral vectors). The initial rationale of the use of C-type retroviral vectors to target exclusively dividing tumour cells on the background of a quiescent tissue is being gradually superseded by the realisation that human tumours generally cycle much more slowly than the rodent cell lines on which this strategy was based.[2] Hence, the trade-off between the total numbers of cells that can be productively infected by an adenovirus, compared with the loss of a potential targeting advantage using C-type retroviruses, clearly favours the more efficient adenoviral system. In addition, the immunogenicity associated with adenoviral vectors probably offers an added 'adjuvant' bonus in the context of most cancer protocols. As a result, the number of direct *in vivo* delivery protocols will continue the escalation of the use of adenoviruses. Of course, the first vector system that comes through with a targeted particle that works in a systemic application may be able to win the initiative back again.[7]

However, even the highest titre system is clearly not high enough yet to cure even local tumours. Therefore, there is a clear need to explore, and exploit, a range of alternative options. Other systems, such as AAV and HSV, are already well developed for use in other gene therapy contexts and may be valuable in certain conditions within the cancer arena.[30] But three areas of intense activity will soon be (1) the development of replicating viruses, (2) the combination of components of different vectors into hybrids with the beneficial properties of different systems and (3) the investigation of novel viral and non-viral delivery systems which have not been explored to their best potential (Figure 1).

The development of replication-competent vectors for cancer gene therapy is the inevitable consequence of data from the early clinical trials. So far, a substantial therapeutic gap still exists between the overlap of the efficacy provided by, on the one hand, the potency of the therapeutic gene(s) and, on the other, the efficiency of gene delivery provided by the vector (Figure 1). Only when these two 'therapeutic domains' approach each other will clinical efficacy result. Therefore, a natural solution to closing this gap is to use viruses that can replicate in tumour cells to enhance gene spread.[31]

The trailblazer of recombinant replication-competent viruses has been the ONYX-015 virus that preferentially replicates in cells lacking functional p53 due to a deletion in the adenovirus E1B gene.[6,11] Phase I and II clinical trials have proved safe enough to allow the virus to go forward for phase III clinical trials – despite some uncertainties as to what exactly is the dependence on p53 status for viral replication.[32] ONYX-015 has no therapeutic transgene itself and relies on the lytic ability of replicating adenovirus to kill cells directly. However, the therapeutic indications from the early trials have again shown that more potency is required over and above that produced by viral lysis of tumour cells and the future development of this system will depend on the incorporation of therapeutic genes into the replicating framework.[33]

As well as other viruses that use intrinsic properties of the transformed phenotype as the basis for tumour-selective replication of a virus,[34] other replication-competent viruses will enter clinical trials based on the use of tumour/tissue-specific transcriptional regulatory elements to drive expression of the critical viral genes required for replication.[35] With a strongly established literature on the use of tissue/tumour-specific promoters within recombinant vectors[36,37] this is a field which is set to proliferate rapidly within the coming years.

An intermediate step on the way to fully replication-competent viruses is the development of hybrid vectors which 'mix and match' elements of established vector systems. Thus, adenoviruses have been used to convert target cells *in vivo* into retroviral producer cells.[38] Similarly, a hybrid between adenovirus and EBV has been described which allows the high titre of adenoviruses to be combined with long-term persistence without integration through the maintenance of a stable EBV episome.[39] In this way, factors such as persistence of expression, titre and immunogenicity of vectors may be controlled more closely than the properties of individual vectors alone can allow.[37]

Finally, the increasing awareness of the potential of gene therapy means that a variety of different viral systems, previously not thought of in the context of vector development, will undoubtedly be developed.[7] Of the large number of viruses that are already well characterised virologically, many have potential for exploitation as vectors. For any given virus, this may either be in its entirety as a novel vector or it may involve cannibalisation of specific components that can be incorporated into other (hybrid) vectors. For example, the VP22 protein of HSV can increase protein distribution between non-transduced cells – thereby enhancing the potential bystander effects.[40] The potential for both well-characterised, as well as lower profile, viral systems to contribute to the recombinant, hybrid and replicating vector pool of the new millennium is, therefore, very great.

Targeting

A *genuine* ability to target delivery systems to tumour cells distributed widely throughout the body of a patient would simultaneously increase real titres and efficacy, and decrease potential toxicity. In truth, no such systemically targeted vectors exist yet. Injection of vectors into the bloodstream for the treatment of cancer requires not only that the vectors be *targeted* (to infect only tumour cells) but also that they be *protected* (from degradation, sequestration or immune attack) for long periods of time so that they can reach the appropriate sites for infection. Moreover, having reached such sites, the vectors must be able to penetrate into the tumour from the bloodstream before carrying out their targeted infection.

Progress in vector targeting has been dramatic in the last few years. Surface targeting would be optimal to prevent non-productive binding and sequestration of vectors before they reach their target cells. It is now possible to activate infection through retroviral envelope binding only in tissues which express, for example, tumour-associated proteases[41] and surface targeting is now also possible for adenoviral vectors.[42] The advent of *in vivo* selection of peptide 'addressin' sequences to target tumour cells or vasculature will add greatly to the technology required to target delivery at the level of cell binding.[43] The challenge will be to show that such addressin peptides can be efficiently and functionally incorporated into vector systems such as viral envelopes. Transcriptional targeting is perhaps more established as a method of limiting gene expression to target cells.[44] However, this approach complements more the *decreased toxicity* of gene therapy rather than contributing to its *increased efficiency*: a transcriptionally targeted vector still has no means of preventing its sequestration by the mass of non-target cells/tissues which it is likely to encounter before it finds its real target. So, despite impressive advances in both promoter design and envelope modification, it still remains to be shown that any of these systems can be used for genuine systemic delivery in which vectors last long enough, and arrive in high enough quantities, at the tumour sites to be effective.

Therefore, the design of most new *in vivo* trials will necessarily have to be based around local delivery, where targeting comes principally through the location of the catheter or syringe needle. As the refinements of vector targeting become more sophisticated, vectors will become available for testing in loco-regional protocols, escalating up through organ/limb perfusion and, eventually, to genuine systemic delivery. However, given the reluctance of the human immune system to permit free circulation of viruses/vectors for any period of time, achieving this latter goal promises to be one of the most difficult of all the challenges to solve.

Anti-angiogenic gene therapy – going for the jugular

Given the difficulties in generating truly targeted vectors for systemic delivery, the alternative is to target those biological properties of tumours which set them apart from all, or most, normal tissues. One of the most notable distinguishing features of tumour growth is the absolute requirement for the tumour to provide itself with an expanding blood supply through the process of angiogenesis, and there is a wealth of targets at the interface between the malignant population and the supporting stroma that could be exploited by approaches including gene therapy.[45] For instance, the migration of tumour endothelium can be inhibited by interfering with protease enzyme function[46] and it may even be possible to design molecules with both antiangiogenic activity and tumour-homing properties.[47] The identification of naturally circulating factors such as angiostatin and endo-

statin, which appear to be capable of suppressing angiogenesis has sparked an explosion in efforts to deliver and express such recombinant molecules[19,47–50] and more candidates are being reported all the time.

Immuno-gene therapy

In principle, the immune system provides exactly what we would like the ideal vector system inherently to provide: (1) an amplification of the therapeutic potential following relatively low level gene delivery, and (2) high level specificity of body-wide target cell killing once correctly activated. The possibility of harnessing these two features to fight metastatic cancer is the reason why the majority of cancer gene therapy protocols have been aimed at immune stimulation (although the cynic might also point out that in the absence of better targeted vectors there has been little other choice!).

Many of the first clinical protocols for cancer gene therapy involved the *ex vivo* modification of freshly isolated tumour cells with cytokines.[10] These trials grew out of safety considerations and a reluctance to deliver viruses directly into tumours *in situ*. However, in the presence of the appropriate *in vivo* controls, it became apparent that in many cases, cytokine modification may be little better than more conventional adjuvant-based cancer cell therapies with no gene transfer component.[51] In addition, the recovery of patient tumour cells and maintenance in culture for long enough to allow transduction with cytokine genes has proved to be laborious and expensive and may significantly alter the phenotype of the cells. None the less, once clear advantages had been shown in animal systems for some cytokines,[52,53] clinical trials have shown encouraging signs that cytokine-modified vaccines can generate significant immune responses in patients with minimal toxicity.[3] However, given the effort and money involved in the autologous cell gene modification approach it is unclear whether these sorts of approaches will ever offer a useful return on the investment.

Perhaps the two most significant areas in which immuno-gene therapy is likely to progress are in the the molecular identification of tumour-associated antigens and exploitation of the central significance of the dendritic cell in generating anti-tumour immune responses.

One of the most spectacular advances during the evolution of gene therapy for cancer has been the cloning of tumour-associated antigens from human tumour (usually melanoma) cells which are recognised by either $CD8^+$,[54] or more recently, $CD4^+$ T cells.[55,56] This has added molecular credibility to the long-held presumption that tumours can indeed express antigens against which T cell-mediated responses can be raised. The tumour vaccination field can now move from the relatively crude level of whole cell vaccines into the molecular arena with defined targets with which to immunise. One cautionary note should be sounded – tumours are highly heterogeneous and unlikely to express only one dominant antigen on all of the cells. Therefore, the trend towards molecular vaccination with defined antigens will undoubtedly have to employ 'cocktail' approaches where multiple cDNAs are used in the vaccination protocol.

With such antigens in hand – even if only in a limited number of tumour types at the moment, the question remains of how to break tolerance to these antigens which are often not even mutated forms of self antigens.

Several key studies have shown that tolerance to tumours can be broken as long as tumour antigens – whether clearly defined or not[57–59] – are delivered into suitably activated dendritic cells.[60,61] Indeed, many of the large numbers of gene transfer experiments which have successfully raised anti-tumour immunity may be attributable simply to the induction of tumour cell killing *in vivo*, leading to transfer of antigens into professional antigen presenting cells (APC).[27,61,62] The efficacy of the resultant anti-tumour responses is likely to be influenced heavily by (1) the efficiency with which DC can be attracted to tumour sites, (2) the availability of tumour antigens to be taken up by DC, and (3) their subsequent maturation/activation and ability to traffic to the lymph nodes to present these antigens.[27] All three of these factors will be encouraged if the tumour cell killing occurs in the presence of pro-inflammatory signals which persuade the immune system to associate a pathological event with the killing.[26] Thus, tumour cells which are killed at low levels by purely apoptotic means are unlikely to (1) attract DC, (2) load the DC or (3) activate the DC. In contrast, cells which die by non-apoptotic means,[23] similar to pathogenic infections, or which die such that the levels of apoptotic death overwhelm the local phagocytic capability to clear them,[59] will stimulate all three of the above. Therefore, we now have the ability to identify the key molecules that can serve as targets for immune responses and to isolate and genetically manipulate the central APC involved in antigen presentation. Coupling these two together means that dendritic cell modification is likely to supplant tumour cell manipulations in the coming years.

It must always be remembered, however, that where immuno-gene therapy for cancer hopes to end up is exactly the point from which gene therapies for autoimmune disease are starting out (Figure 2). Oncologists using immuno-gene therapies seek to induce autoimmunity to a particular class of cells within the patient – their tumour cells. The induction of autoimmunity to some of these tumour-associated antigens has already been shown and correlates well with the generation of anti-tumour immune responses.[63–66] However, the same

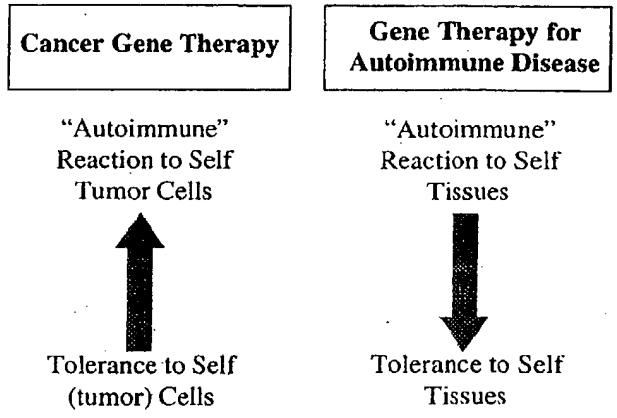

Figure 2 Coming at similar problems from opposite viewpoints. Whereas cancer gene therapists aim to re-educate the immune system to recognise (self) tumour cells expressing tumour antigens, gene therapists treating autoimmune disease aim to prevent the immune system from recognising self tissues.

or related antigens may be displayed on other cells or tissues within the body, differing only in relative expression level, and hence may also represent a target for recognition and destruction. Such side-effects can be tolerated for the treatment of tumours where the normal tissue type is not crucial to survival of the patient (such as melanocytes). However, as immunotherapies for the common cancers of more 'important' tissues develop, the successful induction of autoimmunity to tumour antigens may be accompanied by the potentially disastrous destruction of uninvolved self tissues. The first goal must be the attainment of anti-tumour immune responses; the second will be to learn how to restrict them to tumour, rather than normal tissues of the same type.

Gene therapy in the clinic – knowing its place

A key factor in ensuring the success of gene therapy will be to develop a clear understanding of how it can best play its role in the clinic. For example, immuno-gene therapies are only ever likely to be effective in clinical situations where patients are at, or have been returned to, a state of low tumour burden and still have effective, functioning immune systems.[67] A consequence of this is that as the early phase I/II trials move ahead in to phase III and IV, it will take some considerable time, and large numbers of patients, to demonstrate their true efficacy. Moreover, gene therapy is likely to be very effective in combination with pre-existing clinical regimens, such as chemotherapy and radiotherapy.[6] A large number of studies are now showing great potential for collaboration between gene therapy and pharmaceutical, immunological and radiotherapeutic disciplines to kill cells more effectively and in greater numbers. It is unlikely, therefore, that gene therapy *alone* will play a curative role in cancer treatments for some years. Its importance as a supporting player is much more likely to establish its sense of worth in the minds of clinicians over the coming years, thereby setting the scene for its ultimate use as a frontline therapy in its own right.

Gene therapy and the free market – putting it all together

Gene therapists aspire to creating the Perfect Vector which, for cancer gene therapy, is likely to be rather more multi-component than in other disease situations. It will probably consist of a coat with molecules that allow tumour cell-specific binding; mechanisms to permit transfer of the genes to the nucleus following penetration of the cell membrane; promoter/enhancer elements which target high levels of expression only in the appropriate cells and, of course, the gene(s) that will ultimately lead to the death of the tumour cells directly and/or indirectly through immune stimulation (Figure 3a). Each of these components should be perfected to give the best possible combinations and therapy for the patient. However, even if it were clear what should be put into this perfect vector, will it be commercially possible to assemble it? Few, if any, laboratories can hope to optimise each component from their own endeavours. Therefore, each of the constituent elements (envelopes, promoters, genes, viral systems) will inevitably be the

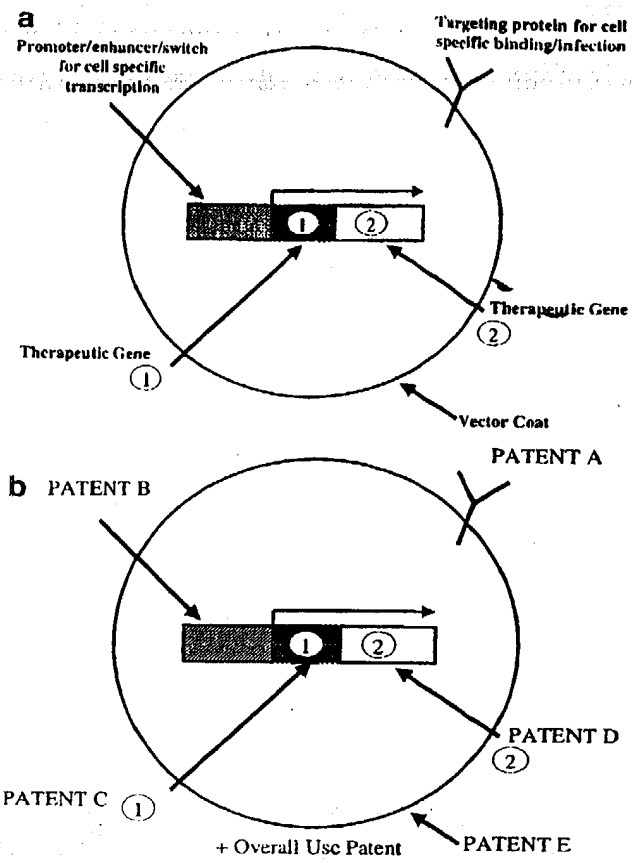

Figure 3 (a) Some of the components of the Perfect Vector of the future, as seen from a research viewpoint. Surface molecules will allow tumour cell-specific binding; promoter/enhancer elements will exclusively target high levels of expression only in the appropriate tumour cells; highly potent, but selective gene(s) will ultimately lead to the death of the tumour cells directly and/or indirectly through immune stimulation. The big question is whether all the separate components, perfected in different laboratories, can interact biologically to generate a truly efficient and targeted vector. (b) The gene therapy vector of the future, as seen from a commercial viewpoint. The big question is whether the patents for all the separate components, owned by a variety of different companies and institutions, can be purchased or licensed at prices that will still allow production, and use, of the vector to be profitable.

intellectual property of different companies or institutions (Figure 3b). What are determined conceptually as the optimal combinations may, in the harsh reality of the free market, at best take long periods of time to co-ordinate and, at worst, never be possible to bring together into one final product. In addition, as ownership of intellectual property begins to dominate what companies are, or are not, prepared to support, finding the funding (and desire) to put together the best possible finished product is going to become increasingly difficult (Figure 3b). It is highly probable that intellectual property priorities are already preventing the initiation of projects which might be the optimal therapeutic priorities, because ownership of certain components of the target vector are not commercially compatible. It will be important to see if the commercialisation of gene therapy can truly complement, rather than corrupt, its therapeutic promise in the new millennium.

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Gene Therapy for Cancer

J. Gómez-Navarro,[1,2] D.T. Curiel[1,3] and J.T. Douglas[1,3]

[1]Gene Therapy Center; [2]Division of Hematology/Oncology; and [3]Division of Pulmonary and Critical Care Medicine, The University of Alabama at Birmingham, 1824 Sixth Avenue South, Birmingham, Alabama 35294-3300, U.S.A

Jesús Gómez-Navarro is a medical oncologist trained at the Hospital La Paz, Universidad Autonoma de Madrid, Madrid, Spain. He received further postdoctoral training in gene therapy at the Gene Therapy Center, University of Alabama at Birmingham (UAB), Birmingham, Alabama, U.S.A., where he is currently a Research Assistant Professor in the Division of Hematology/Oncology.

David T. Curiel is Director of the Gene Therapy Center at the University of Alabama at Birmingham (UAB), Alabama, U.S.A. He is also Professor of Medicine and Jeanne and Anne Griffin Chair for Women's Cancer Research at UAB.

Joanne T. Douglas received postdoctoral training in the Gene Therapy Center, University of Alabama at Birmingham, Alabama, U.S.A., where she is currently a Research Assistant Professor in the Department of Medicine.

Correspondence to D.T. Curiel, e-mail: david.curiel@ccc.uab.edu
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WHY GENE THERAPY?

It is well established that most cancers result from a series of accumulated, acquired genetic lesions in somatic cells that are faithfully reproduced until a malignant clone is created, which is ultimately able to destroy the host. To a larger and larger extent, the genetic lesions associated with malignant transformation and progression in a wide variety of human cancers are being identified [1, 2]. Armed with this knowledge of the molecular anatomy of the cancer cell, gene therapy has emerged as a new method of therapeutic and possibly preventive intervention against cancer targeted at the level of cellular gene expression [3]. In this approach, the complex cancerous pathophysiological state is altered by delivering nucleic acids into tumoral or normal cells. These nucleic acids may be genes, portions of genes, oligonucleotides, or RNA. In conventional therapeutics, as in pharmacotherapy, a cell or tissue phenotype is altered by modifying cell physiology or metabolism at the level of protein expression. In contrast, in gene therapy this is accomplished by changing the pattern of expression of genes whose products may thus achieve the desired effect on the cellular phenotype.

In the treatment of human disease, gene therapy strategies may offer the potential to achieve a much higher level of specificity of action than conventional drug therapeutics by virtue of the highly specific control and regulatory mechanisms of gene expression that may be targeted. Additionally, interceding at an earlier, upstream step in disease pathogenesis may offer greater potential to induce fundamental changes in phenotypic parameters of disease, with a more favourable clinical outcome. The availability of gene transfer systems, or vectors, for permanent or long-term genetic modification of cells and tissues should allow definitive therapeutic or preventive interventions. Furthermore, gene transfer may be accomplished in a limited loco-regional context, producing a high concentration of therapeutic molecules in the local area. Thus, undesired systemic effects of those therapeutic molecules are avoided. Lastly, using the body to produce therapeutic proteins, potentially in only certain tissues, has practical advantages of its own [4]. Briefly, limitations associated with manufacture, stability, and duration of effect after administration of drugs based on synthetic peptides are completely avoided. From the same pharmacological point of view, designer drugs based on small molecules, currently under intensive investigation, can hardly substitute the function of complex defective proteins, such as many products of tumour suppressor genes.

In the treatment of human malignant tumours, several obstacles explain the limitations of currently available treatments for achieving definitive cures in most cases of advanced disease (Table 1). It is apparent that a combination of new chemotherapy drugs, higher doses of drugs, novel cytokines, improved regimens of radiotherapy, and more sophisticated surgery can achieve incremental improvements in cancer treatment. But these therapies do not address critical biological obstacles and, thus, probably will not bring the much-needed radical advances in the implementation and results of cancer treatment. In contrast, gene therapy offers the potential for overcoming some of these fundamental barriers (Table 1).

Table 1. Potential contributions of gene therapy to overcome obstacles for curing cancer

Obstacles to curing cancer	Potential contribution of gene transfer
1. All tumours are *genetically unstable* and thus they are extraordinarily adaptible to environmental changes.	Gene transfer of DNA repair or cell cycle checkpoint genes that restore DNA stability and cell susceptibility to therapeutic insults.
2. Tumours are heterogeneous in many respects, including genetic mutations, expression of oncoproteins, immunogenicity, response to environmental changes, etc.	Targeting of genetically homogeneous and stable tumors, such as the tumour vasculature and stroma; genetic immunopotentiation; chimeric vectors.
3. As a consequence of obstacles 1 and 2, tumours have or acquire *resistance* to cellular toxins and to many other therapeutically induced cellular insults.	Strategies above, associated with chemotherapy or radiotherapy, or with the transfer of additional genes that sensitize tumour cells to drugs or radiation.
4. Tumours can have a low *cellular growth fraction*; therefore, they are less susceptible to mitotic toxins and to gene transfer vectors that require dividing cells.	Use of vectors that do not require cellular division for gene delivery and expression (adenovirus, herpesvirus, lentivirus, chimeric vectors); repeated administration of non-immunogenic vectors.
5. Tumours form *metastases*, which have to be reached systemically to eradicate the tumour completely.	Use of targetable, injectable vectors (tropism-modified viruses, cellular vehicles, liposomes); genetic immunopotentiation.
6. Tumours do not express specific tumour antigens or immune costimulatory molecules; alternatively, tumours down-regulate antigen-presentation, induce *immunological tolerance*, or inhibit the effector mechanisms of the immune response.	Transfer of genes encoding costimulatory molecules and cytokines; genetic modification of antigen-presenting cells; induce inflammatory reactions that activate antigen presentation; transfer of genes blocking tumour-secreted inhibitors of the immune response.
7. The *spontaneous* behaviour of human tumours is somewhat different from that of malignant cells *in vitro*, and from that of experimental tumours in animal models.	Development of better animal models, including tumour models in transgenic mice.
8. Tumours are diagnosed in *advanced stages*, when billions of tumour cells exist in the body, frequently widely disseminated.	Development of amplification vector systems (replicative viral vectors and exploitation of bystander effects); use of targetable, injectable vectors; genetic immunopotentiation.
9. The understanding and treatment of cancer requires the contribution of very *diverse fields* of basic knowledge, biotechnology, and medical practice.	*De facto* multidisciplinary recruitment of gene therapy researchers.

STRATEGIES

A number of strategies have been developed to accomplish cancer gene therapy. These approaches include (1) mutation compensation, (2) molecular chemotherapy, and (3) genetic immunopotentiation. For mutation compensation, gene therapy techniques are designed to correct the molecular lesions that are aetiologic of malignant transformation, or avoid the contribution to malignant growth by tumour-supporting non-malignant stromal cells. For molecular chemotherapy, methods have been developed to achieve selective delivery or expression of a toxin gene in cancer or stromal cells to induce their eradication, or alternatively to increase their sensitivity to concomitant chemotherapy or radiotherapy. Also, attempts have been made to deliver genetic sequences that protect normal bone marrow cells from the toxic effects of standard chemotherapeutic drugs, thus allowing the administration of higher drug doses without reaching otherwise limiting myelosuppression. Genetic immunopotentiation strategies attempt to achieve active immunisation against tumour-associated antigens by gene transfer methodologies. Both tumour cells and cellular components of the immune system have been genetically modified to this end. Importantly, each of these approaches has been rapidly translated into human gene therapy clinical trials [5] as summarised in Table 2.

In this review, we examine the lessons learned from the results of the first attempts to apply each gene therapy strategy in human cancers. In each section, we show both the rationale of gene therapy and the problems encountered in its development, emphasising the general biological concepts of each therapeutic strategy. Finally, we illustrate prospects for overcoming the obstacles to implementation of gene therapy by novel methods that are currently being refined.

MUTATION COMPENSATION

The knowledge of the major role that growth factors, signalling molecules, cell cycle regulators, and determinant factors of angiogenesis, invasiveness and metastasis play in neoplastic progression has positive implications for gene therapy. That is, it is possible to abrogate the malignant phenotype by correcting the underexpression of tumour suppressor genes or overexpression of oncogenes involved in these phenomena. At the level of the single cell, the inactivation of tumour suppressor genes contributes to the neoplastic phenotype by abrogating critical cell cycle checkpoints, DNA repair mechanisms, and pro-apoptotic controls. To approach this loss of function, the logical intervention is replacement of the deficient function with the wild-type gene. Mutations of more than 24 tumour suppressor genes have been described in numerous cancers. Of these, *p53*, *RB1*, and *BRCA1* are currently being administered in clinical trials as replacements for the mutated counterparts (Table 3). In all these cases, pre-clinical studies showed some expression of the wild-type protein after gene delivery and reversion of the malignant phenotype, frequently associated with the induction of apoptosis in tumour cells [6]. Of note, however, some tumours have shown persistent tumorigenicity and proliferation after successful restoration and expression of wild-type genes, a phenomenon referred to as 'tumour suppressor resistance'. Other major obstacles are mentioned later.

For dominant oncogenes, it is the aberrant expression of the corresponding gene product that elicits the associated neoplastic transformation. In this context, the molecular therapeutic intervention is designed to ablate expression of the dominant oncogene. Inhibition of oncogenic function can be attempted at three levels. First, transcription of the oncogene can be inhibited. This approach uses triplex-forming oligonucleotides or other sequences that bind transcriptional start sites in the genomic DNA. An example, currently being clinically tested, is based on the adenoviral gene E1A, that inhibits transcription of the human c-*erbB-2* promoter and accordingly suppresses the tumorigenicity and metastatic potential induced by the *erbB-2* oncogene. Second, translation of the oncogene messenger RNA can be blocked using specific antisense sequences, which function by promoting degradation of the complementary message [7]. Evidence for a specific effect of antisense molecules has been particularly compelling in selected cases, and these molecules are currently undergoing clinical tests. These include antisense sequences against insulin-like growth factor 1 in glioma, K-*ras* in lung cancer, *c-myc* in breast and prostate cancer, and TGFβ in glioma (Table 3). Practical constraints have limited wide employment of this technology in protocols of human anticancer gene therapy, including the idiosyncratic efficacy of specific antisense for a given target gene and the suboptimal delivery of antisense molecules. Third, mobilisation of the nascent oncoprotein can be blocked or its function can be inhibited when in its final cell location. These last strategies involve the use of 'intracellular antibodies' that intercept and interfere with the intracellular processing of the oncoprotein, or the heterologous expression of mutant proteins that can inhibit the function of the native oncoprotein,

Table 2. Clinical trials of gene therapy for the treatment of cancer

Strategy	Clinical trials*	Molecular mechanism of antiCancer effect
Mutation compensation	76	Inhibition of expression of dominant oncogenes
	18	Augmentation of deficient tumour suppressor genes
	2	Abrogation of autocrine growth factor loops (single chain antibodies)
Molecular chemotherapy	27	Selective delivery of toxin or toxin gene to cancer cells
	9	Chemoprotection of normal tissues during high-dose chemotherapy
Genetic immunopotentiation	54	*In vitro* transduction—augmentation of tropism or cell killing capacity of tumour-infiltrating lymphocytes; genetic modification of irradiated tumour cells
	47	*In vivo* transduction—administration of costimulatory molecules or cytokines; immunisation with virus encoding tumour-associated antigens
Viral-mediated oncolysis	2	Tumour cell lysis by viral vector replication

*Registered in the NIH Office of Recombinant DNA Activities in December 1998 (http://www.nih.gov/od/orda/protocol.pdf).

Table 1. Molecular manipulation targeted endothelially

Target	Strategy	Vector	Tumour type
p53	Replacement of tumour suppressor gene	Adenovirus	Non-small cell lung cancer, head and neck squamous cell carcinoma, hepatic metastases of colon cancer, hepatocellular carcinoma, prostate cancer, breast cancer
RB (Retinoblastoma)	Replacement of tumour suppressor gene	Adenovirus	Bladder cancer
BRCA-1	Replacement of tumour suppressor gene	Retrovirus	Ovarian cancer
erbB-2	Inhibition of promoter by E1A	Cationic liposome complex	Breast and ovarian cancers overexpressing erbB-2
Insulin-like growth factor I	Blockade by antisense	Cationic liposome complex	Glioblastomas
k-ras	Blockade by antisense	Retrovirus	Non-small cell lung cancer
c-myc, c-fos	Blockade by antisense	Retrovirus	Breast and prostate cancers
TGFβ	Blockade by antisense	Plasmid and electroporation	Glioblastoma
erbB-2	scFv	Adenovirus	Ovarian cancer

*Registered in the NIH Office of Recombinant DNA Activities December of 1998 (http://www.nih.gov/od/orda/protocol.htm) scFv: single-chain intracellular antibody.

respectively. We have shown, for instance, that intracellular expression of an anti-erbB-2 single-chain antibody (scFv) results in down-regulation of cell surface erbB-2 expression and selective cytotoxicity in tumour cells expressing the oncogene target both *in vitro* and *in vivo* [8, 9].

TUMOUR PHENOMENA DEPENDENT ON MULTIPLE GENES

Angiogenesis

The development of new blood vessels is a critical step in the growth, progression, and metastatic spread of both solid and haematopoietic tumours. Despite heterogeneity in many other respects, all tumours thus share a universal feature, i.e. they depend absolutely on the vasculature not only to sustain their initial growth and dissemination but also to maintain their long-term viability. Extensive experimental data and clinical-pathological studies support this contention (reviewed in: Special Issue on Angiogenesis, *European Journal of Cancer*, Vol. 32, issue 14, 1996). Vessel targeting, therefore, should be useful for the treatment of most kinds of cancer, either to impede the formation of tumour blood vessels, as initially proposed by Folkman (see Special Issue), or as an attempt to destroy the already formed tumour vasculature, as proposed later by Denekamp and others [10–12]. From the points of view of oncology and gene therapy, two features of this strategy seem most attractive. First, the genetic stability of endothelial cells should essentially eliminate the appearance of resistance to molecular therapeutic interventions [13], which is so pervasive in the treatment of tumour cells. This hypothesis has been confirmed in a cancer animal model that evaluated treatment with the natural angiogenesis inhibitor endostatin [14]. Second, an additional advantage of targeted killing of endothelial cells is the highly amplified killing effect of large numbers of tumour cells when deprived of their vascularisation. This can partially overcome current limitations in the number of cells modified by gene transfer *in vivo*.

In the last decade, anti-angiogenic drugs targeted to the proliferating endothelium of tumours and other diseases have been applied in the clinical setting and have entered clinical trials. In addition, the association of chemo- or radiotherapy with anti-angiogenic agents has been shown to produce an enhanced anti-tumour effect in preclinical models. Notably, combined treatments can achieve cures that are not observed with either treatment alone [15]. Thus, molecular therapeutic interventions against the tumour and its vasculature are not only strongly appealing on theoretical grounds for their use in a variety of clinical contexts, but their utility is also rapidly being tested clinically [16]. Based on this, genetic modification of the endothelium of tumour vasculature has been proposed as an alternative therapeutic modality [17, 18]. With this genetic strategy, the problems of previously explored approaches can be potentially overcome. For instance, local production of high levels of therapeutic proteins can be induced, thus obviating or diminishing the difficulties associated with systemic toxicity, and also pharmacological issues, such as largescale manufacture, bioavailability, and cost of ordinary drugs. In addition, the ability to release the gene product continuously may be relevant in certain cases, such as for the appropriate anti-angiogenic effect of interferon gamma.

Both suppression of angiogenic cellular signals and augmentation of natural inhibitors of angiogenesis have proved to be feasible strategies in *in vivo* tumour models. Examples of effective genetic interventions for the suppression of angiogenesis factors are the down-regulation of vascular endothelial growth factor (VEGF) by antisense molecules, as shown in models of glioma [19, 20], and the blockade of VEGF receptor function by delivery of mutant versions of one of its cognate membrane receptors, Flk-1 [21, 22], or of a secreted soluble version of its other receptor, Flt-1 [23, 24]. In addition, similar results have been obtained by adenoviral-mediated delivery of a soluble receptor analogous to the endothelium-specific Tie2 receptor [25], also known to play a role in tumour angiogenesis. Conversely, the replacement or supplementation of inhibitors of angiogenesis has been attempted by transfecting cells with the thrombospondin gene [26] and by using *in vivo* viral vectors that encode soluble platelet factor 4 [27] and angiostatin [28, 29]. However,

none of these strategies has been clinically tested and major issues, mostly vectorological, are still to be solved. Most obvious are the problems of assuring highly efficient gene delivery and long-term expression of the therapeutic anti-angiogenic genes to keep the tumour deprived of its growth-enabling vascularisation. In addition, the current lack of targetable, injectable vectors impedes the application of anti-angiogenesis gene-based strategies to multiple foci of tumour that characterise disseminated cancer. Lastly, different combinations of endothelial growth factors and their receptors are altered in different tumours, and may even change in single tumours during different stages of progression. Thus, despite its powerful rationale, the successful clinical implementation of anti-angiogenesis gene therapy will still require major developments.

Anti-angiogenesis seems a therapeutic manoeuvre mostly appropriate for avoiding tumour progression but, as mentioned above, alternative anti-vascular strategies have been proposed with the intention of destroying existing vasculature, thereby depriving the tumour of essential vascularisation. To date, there have been few attempts to induce direct toxicity in the vasculature of normal or tumour vasculature by gene transfer [30, 31], but development of targeted vectors should prompt immediate evaluation of such strategies.

Invasion and metastasis

Increasingly, genes and proteins involved in phenotypic aspects of tumours, other than disordered proliferation, are being described and identified as potentially useful therapeutic targets. In this regard, besides angiogenesis, one fundamental component of the metastatic cascade is the local invasion of the extracellular matrix by tumour cells. Studies in animal models have begun to show that modulation by gene transfer of molecules involved in degradation of extracellular matrix, cellular motility, and cellular adhesion, such as plasminogen activators, metalloproteinases and CD44, has the potential for inhibiting tumour cell spread [32]. To have clinical utility, however, these manoeuvres should provide long-term abrogation of the involved molecules and be used when the tumour is going through the earlier steps of the metastatic cascade in a particular patient.

Apoptosis

The highly orchestrated form of cell death known as apoptosis goes awry to some extent in most cancers. Increasingly, a general theme in cancer pathophysiology is the development of a defect in the function of pro-apoptotic molecules, such as p53, that commonly prepare the cell for apoptosis whenever cell proliferation or DNA damage is induced, their absence thus depriving the cell of a critical safety mechanism [33]. Alternatively, a functional excess of anti-apoptotic molecules, such as Bcl-2, may also occur in tumours. In each case, the result is an imbalance that favours the inappropriate survival of tumour cells. The mechanisms involved are attractive therapeutic targets because the tumour cell is totally dependent on them for its survival, and appears to have a higher sensitivity to the induction of apoptosis than normal tissues [33]. In addition, restoring or enhancing the capacity to undergo apoptosis may, in some cases, be a crucial event which renders tumours sensitive to classical anticancer agents, such as chemotherapy [34, 35] and radiotherapy [36, 37].

With the increasing recognition of the molecular basis of the apoptotic pathway [33, 38–40], and the description of several of its components acting as oncogenes or tumour suppressor genes, gene therapy has emerged as a rational strategy for the modulation of apoptosis. Therefore, the genetic modification of tumour cells and their supportive stroma with genes that modulate the apoptotic process has been recently proposed for the gene therapy of cancer [6, 41–43]. Three general requirements for the successful therapeutic application of genetic modulation of apoptosis in cancers are apparent. First, significantly better gene transfer vectors may be needed to modify and trigger apoptosis in most malignant cells in any given tumour. Current vectors are far from achieving *in vivo* the requisite high levels of tumour cell modification. Alternatively, mechanisms may be implemented regionally to amplify the effects of the expression of transferred genes, i.e. by inducing a bystander apoptosis. Second, given the ubiquity of the numerous cellular proteins involved in apoptotic pathways, selective activation in cancer cells of the lethal processes may also be a critical requirement of therapeutic manoeuvres. The lower threshold for undergoing apoptosis that characterises tumour cells could, however, offer an advantageous therapeutic window that makes this requirement less stringent. Third, given the complexity and redundancy of the signalling circuits involved, modulation of several components of the apoptotic pathways may be needed to provoke cell death. Interventions downstream in the circuits might also be preferable to avoid regulatory counterbalances.

Despite the theoretical constraints just mentioned, preliminary attempts to explore the therapeutic modulation of apoptosis against cancer by gene transfer have already begun, driven by encouraging preclinical data in animal models. Clinical trials are currently ongoing evaluating the value of pro-apoptotic *p53* and adenoviral E1A, and a growing number of other candidate genes are being considered and tested preclinically (Table 4).

Obstacles to mutation compensation

Although the strategies currently used for the restoration of normal genes and ablation of mutant genes have offered in-depth insights into the molecular biology of carcinogenesis and tumour progression, they face critical problems that restrict their clinical application. Human tumours are remarkably heterogeneous in the patterns of expression of relevant oncogenes. Thus, therapeutic targeting of a single molecular abnormality may have only an inconsequential impact on the clinical management of the disease, considering both the population and individual patients. In addition, several mutated genes produce molecules with transdominant effects, thus necessitating the blocking of their effects and not merely their supplementation with a wild-type version of the gene. Furthermore, because these strategies mostly modulate intracellular responses, nearly every tumour cell might have to be targeted for these approaches to be clinically effective. The current state of development of gene therapy vectors, both viral and non-viral, makes this feat unachievable within non-toxic margins of vector dose. Clearly, breakthrough developments in vector technology are needed for these obstacles to be overcome. A better understanding of the tumour-supportive micro-environment and of multicellular tumour phenomena may also suggest genetic interventions that, even with a limited gene transfer, can elicit widespread effects in the tumour. In addition, approaches such as molecular chemotherapy or immune system augmentation that exhibit an amplified regional or systemic effect hold the promise of tackling some of the aforementioned limitations.

Table 2. Genetic manipulation of apoptosis for cancer therapy

Strategy	Target genes or molecules
Add or restore pro-apoptotic molecules	
Induce exogenous death signals (ligand/death receptor)	Granzyme B/Granta 519, FasL (CD95L)/Fas (CD95), TNF/TNFR1, Apo2L/DR5, Apo2L/TRAIL/DR4 or DR5
Induce endogenous triggers of apoptosis	Cytochrome c, TP53, E1A
Induce pro-apoptotic regulators	bax, bcl-xs, apoptin
Re-link pro-apoptotic signals with apoptosis effectors	Apoptosome components (cytochrome c, Apaf-1, CARD, caspase-9), others
Activate directly apoptosis effectors	Caspase recruitment domain (CARD)
Suppress anti-apoptotic molecules	
Inhibit exogenous survival signals	?
Inhibit inhibitors of exogenous death signals	NF-κB
Inhibit anti-apoptosis regulators	bcl-2
	Inhibitors of apoptosis (IAPs), such as survivin, XIAP, IAP-1, and IAP-2

Genes used or targeted in clinical trials appear in italics.

MOLECULAR CHEMOTHERAPY

A number of distinct approaches to molecular chemotherapy for cancer have been developed. These include the administration of (1) toxin genes to eliminate tumour cells and the stromal cells that support them, (2) drug resistance genes to protect the bone marrow from myelosuppression induced by chemotherapy, and (3) genes that enhance the effect of conventional anticancer treatments. Initially, the approach of molecular chemotherapy was designed to achieve selective eradication of carcinoma cells via expression of a toxin gene. This is similar to conventional chemotherapy, where pharmacological agents are employed. However, in the latter approach, toxicity of the drug is often manifested both in malignant and non-malignant cells. Therefore, in order to effect a reduction in the burden of neoplastic cells, the patient's normal tissues and organs have to be exposed to potentially harmful quantities of the drug. Molecular chemotherapy is designed to circumvent this limitation by selectively targeting toxin delivery or expression to cancer cells on the basis of more specific tissue- or transformation-associated markers, thereby reducing the potential for non-specific toxicity. Commonly, a non-toxic pro-drug is administered that requires activation in genetically modified cells in order to be transformed into a toxic metabolite that ultimately leads to cell death [44–49].

Toxin genes

Thymidine kinase. The most common molecular chemotherapy system utilised to date to accomplish cell killing has been the herpes simplex virus thymidine kinase (HSV-*tk*) gene given in combination with the pro-drug ganciclovir (GCV) [50]. The selectivity of the HSV-*tk* system is based on the fact that, contrary to normal mammalian thymidine kinase, HSV-*tk* preferentially monophosphorylates GCV, rendering it toxic to the cell. GCV is then further phosphorylated by cellular kinases to produce triphosphates that are incorporated into cellular DNA. The incorporation of the triphosphate form of GCV causes inhibition of DNA synthesis and of RNA polymerase, leading to cell death [44]. Thus, tumour cells (or any other cell undergoing mitosis) transduced to express the viral *tk* gene have enhanced sensitivity to cell killing after exposure to GCV. Somewhat unexpectedly, normal cells transduced with HSV-*tk* after intravenous (i.v.) [51] or intrahepatic [52] administration of adenoviral HSV-*tk* vector have also shown high sensitivity to GCV, leading to liver degeneration and low survival in mice. The absence of toxicity of GCV after i.v. administration of a control adenovirus or subcutaneous administration of an adenovirus encoding HSV-*tk* suggests that the toxicity is specifically liver-associated. The relationship between toxicity and the status of liver parenchymal cells with respect to the cell cycle remains to be determined. The toxicity and efficacy of the transfer of HSV-*tk* are currently being tested in more than two dozen phase I human clinical trials, including tumours of the ovary, brain, prostate, head and neck, mesothelioma, multiple myeloma, leukaemia, and liver metastasis of colon cancer (for an updated list of protocols visit the Office of Recombinant DNA activities website at http://www.nih.gov/od/orda/protocol.htm).

Bystander effect. Whilst the benefits of selectively eradicating tumour cells are obvious, an important limitation associated with molecular chemotherapy is the inability to genetically modify 100% of the tumour cells with the toxin gene. However, this has proved not to be as severe a limitation as initially thought due to the phenomenon known as the 'bystander effect', whereby the eradication of HSV-*tk* transduced cells elicits a killing effect upon the surrounding non-transduced tumour cells. That not all of the tumour cells need to contain the HSV-*tk* gene to obtain complete eradication of the tumour was an observation of early experiments employing the relatively inefficient retroviral vectors in brain tumours [53, 54]. This occurrence was later confirmed in a variety of other tumour model systems [55–58].

Other toxins. Several additional combinations of enzyme/pro-drug have been developed to improve the efficacy of molecular chemotherapy and overcome the limitations of *tk*/GCV. For example, some of the enzyme/pro-drug combinations induce toxic effects not only in cycling but also in non-cycling cells (carboxypeptidase G2, nitroreductase, purine nucleoside phosphorylase). With others, the bystander effect is stronger (purine nucleoside phosphorylase) or does not require cell contact (cytosine deaminase, nitroreductase).

With some exceptions, single drugs in standard chemotherapy do not cure cancer. Historically, effective cancer treatments were developed when drugs with different mechanisms of action were used in combination. Extending

this concept to molecular chemotherapy, several combinations of enzyme/pro-drug have been shown to induce synergistic killing effects *in vivo* [59, 60]. Combination schemes have achieved also higher rates of tumour regression and cure in animal models [61, 62]. Thus, the application of classical chemotherapy principles for designing drug combinations would recommend the use of pro-drug/enzymes that target both dividing and non-dividing cells, that elicit different mechanisms of bystander effect, and that have non-overlapping toxicities.

Drug-resistance genes

In a second molecular chemotherapy approach, the host tolerance to higher doses of standard chemotherapeutic drugs is increased by transducing bone marrow cells, known to be highly sensitive to chemotoxicity, with genes that confer drug resistance [63–65]. Some potential problems with this strategy are, however, apparent. These include the absence of clear cut evidence demonstrating that higher chemotherapy doses translate into improved patient survival, very low transduction efficiency of the target human haematopoietic cells with retrovirus vectors, the dose-limiting effects determined by other non-haematological toxicities, and the fact that contaminating cancer cells in the marrow could be transduced with the drug-resistance gene, which could rapidly give rise to clones of treatment-resistant tumour cells.

Chemosensitisation and radiosensitisation

A third approach of molecular chemotherapy seeks to modulate the level of expression of a variety of genes that influence the sensitivity of the cell to toxic stimuli, including conventional chemotherapeutic drugs and radiotherapy. Genetic chemosensitisation can be achieved by inducing apoptosis, by inhibiting molecules involved in tumour cell resistance, or by enhancing intratumoral production of cytotoxic drugs. To facilitate apoptosis, genes such as *p53* may be administered to tumour cells to enhance the mechanisms of apoptosis induced by chemotherapeutic agents [66]. Our group has shown that down-regulation of Bcl-2 protein levels by an intracellular anti-Bcl-2 single-chain antibody increases drug-induced cytotoxicity [67]. Analogously, genetic down-regulation of cellular factors related to chemoresistance has been shown to enhance chemosensitivity [68]. Alternatively, genes can be administered intratumorally to enhance metabolic conversion of conventional chemotherapeutic agents. For example, transfer of a liver cytochrome P450 gene, *CYP2B1*, into human breast cancer cells greatly sensitised these cells to the cancer chemotherapeutic agent cyclophosphamide as a consequence of the acquired capacity for intratumoral drug activation. This effect produced a substantially enhanced antitumour activity *in vivo* [69]. Lastly, combinations of conventional chemotherapeutic agents and molecular chemotherapy can serve the established rule of administering cytotoxic drugs with different mechanisms of action and toxicities. For example, one ongoing clinical trial is evaluating the association of adenovirus-mediated transduction of ovarian cancer cells with the *tk* gene followed by administration of acyclovir and the chemotherapeutic drug topotecan (http://www.nih.gov/od/orda/protocol.pdf).

Several drugs are proven radiosensitisers, a fact that is commonly exploited in the clinic. One of these drugs is 5-fluorouracil (5-FU), which can be produced by the cytosine deaminase (CD) suicide gene. In this regard, molecular chemotherapy based on CD has been shown to enhance the effects of radiation therapy in animal models of gliosarcoma and cholangiocarcinoma [70]. Thus, strategies to alter both chemosensitivity and radiosensitivity by gene transfer appear to have potentially wide applicability in many tumour contexts.

Obstacles to molecular chemotherapy. With all its promise, molecular chemotherapy also bears some practical limitations. To date, the strategy of molecular chemotherapy has been mainly used in loco-regional disease models to overcome the lack of targeted vector systems. In these *in situ* schemes, a vector encoding the toxin gene is administered intratumorally or into an anatomic compartment containing the tumour mass. The goals of this delivery method are to achieve high local vector concentration in order to favour tumour transduction and to limit vector dissemination. However, transduction efficiencies of presently available vectors have been shown to be inadequate. Even in the context of closed compartment delivery, it has not been possible to modify a sufficient number of tumour cells to achieve a clinically relevant tumoral response [71]. Furthermore, although transduction with HSV-*tk* followed by ganciclovir treatment reduces tumour burden and prolongs survival in various model systems, including those utilising intratumoral and intraperitoneal (i.p.) administration, the elevated doses of viral vector needed to obtain transduction of the majority of the tumour cells are associated with limiting toxicity. In fact, substantial toxicity and experimental animal death have been noted [51, 52, 72]. Thus, the small therapeutic index of currently available vectors in the context of *in situ* administration is a critical limiting factor for the purpose of gene therapy of cancer. Furthermore, and most importantly, a well-known limitation of conventional chemotherapy is also to be expected with the use of molecular chemotherapy, i.e. the appearance of drug-resistant tumour subpopulations (Table 1). In conclusion, vector limitations and well-known barriers to classical cytotoxic manoeuvres impede the full exploitation of the promise of a more selective eradication of carcinoma cells via the expression of toxin or protective genes.

GENETIC IMMUNOPOTENTIATION

The development of clinically evident tumours implies the obvious failure of the host immune system to recognise and eliminate tumour antigen(s), a hypothetical role suggested by Thomas and embodied by Burnet under the name of 'immune surveillance of neoplasia' [73]. Genetic immunopotentiation strategies attempt to achieve active immunisation against tumour-associated antigens by gene transfer methodologies applied either to tumour cells, to enhance their immunogenicity, or to cellular components of the immune system, to enhance their anti-tumour prowess.

Genetic modification of immune effector cells

Cells of the immune system have been modified to augment their capacity to recognise and reject tumour antigens [74]. To this end, gene therapy offers the possibility of genetically modifying effector cells and, importantly, this intervention can be performed *ex vivo*, thus avoiding the toxicity that characterises most biological response modifiers when administered systemically.

Tumour infiltrating lymphocytes (TILs). TILs are derived from mononuclear cells obtained from leucocytes infiltrating resected specimens of solid tumours. In the early 1990s, it was hypothesised that TILs could be an enriched source of

natural killer (NK) cells and cytotoxic T-lyphocytes (CTLs) specific for tumour antigens, and could also have tropism towards systemic tumour foci. On this basis, technology for their expansion in culture was developed, and TILs were the first immune cells to be genetically modified and applied in a human gene therapy clinical trial against cancer [75]. It was soon observed that while TILs do include CTL and NK activated cells, only a few of these cells in these mixed populations are specific against the tumour from which they are isolated. Furthermore, reinfused TILs localised poorly into tumours, and their required expansion *in vivo* using IL-2 was rather toxic. Although several strategies have been applied to improve treatments based on TILs and other lymphocytes, including an elegant re-engineering of their tropism [76, 77], a modest localisation of TILs in tumours remains a limitation for the efficacy of this poorly tolerated and expensive therapy.

Genetic modification of tumour cells

An alternative strategy for trying to augment the anti-tumour immune response is to genetically modify tumour cells, or to manipulate their components, to facilitate the start of a robust immune response. Thus, it has been hypothesised that a formerly tolerant host may revert its immune status, characterised by tolerance or anergy, and thus ultimately experience tumour rejection. In other words, it is hypothesised that the host can be 'vaccinated' against the tumour by exposing tumour antigens to the immune system in a more favourable context [78–81]. Most clinical experience with antitumour vaccines to date has been obtained in melanoma patients. For years, irradiated tumour cells, either autologous or allogeneic, were administered in combination with different adjuvants, such as BCG. Later, the molecular definition of tumour-associated antigens allowed the testing of vaccines based on individual antigenic determinants delivered to the patient in the form of peptides or DNA. More recently, tumour cells themselves have been genetically modified to increase their immunogenicity by transfer of a variety of genes, including cytokines such as GM-CSF, costimulatory molecules such as B7, and MHC molecules. Clinical responses have been occasionally observed in melanoma, but not in colon or renal cancer [82]. A common requirement, not adequately accomplished routinely yet, is to introduce the gene of interest in tumour explants or cultured cells with high efficiency. A more fundamental problem has been observed in experimental models using tumours naturally arising in transgenic mice. In these spontaneous tumours, a clear lack of efficacy of vaccines called into question the relevance of previously observed responses in animal models of grafted, syngeneic tumours [83].

Obstacles to genetic immunopotentiation

The main advantage of genetic immunopotentiation is the possibility of enlisting physiological mechanisms for a potentially vast amplification of the therapeutic manoeuvre. To this end, even modest levels of gene transfer were initially expected to be followed by clonal expansion and systemic spread of effector immune cells and mediators. Thus, efficiency of gene transfer would be not critical, given the relatively low amounts of cells and gene products needed to obtain a potentially powerful response from the immune system.

The level of gene transfer into tumour and immune effector cells observed clinically has been limited [82], and this has been thought to partly explain the poor results obtained by tumour immunotherapy in humans. However, there are other, probably more important, obstacles. Factors that can explain the failure of the immune system in the cancer patient are legion, and it is not clear which of them are critical in the clinical context. Some of these factors may similarly explain the failure of previous immune therapeutic attempts. In general, a lack of an immune response can be due to inadequate immunogenicity of the tumour or to a deficiency of the immune system to recognise, respond and reject tumour antigens. Reduced tumour immunogenicity can be related to the absence, heterogeneity and plasticity of tumour-specific antigens or the loss of MHC class I molecules on the tumour cells, which are essential for presentation of cellular antigens to effector $CD8^+$ T lymphocytes. Alternatively, it may well be that the lack of costimulatory molecules, such as B7, in tumour cells and the lack of other 'danger' signals in the tumour site establishes immune tolerance or ignorance, which keeps the tumour from being rejected. In effect, current knowledge of tumour immunobiology establishes that T cells able to recognise tumour-associated antigens can be found *in vivo* and are inducible *in vitro*. Thus, the lymphocyte repertoire against these epitopes has not been deleted. However, either tolerance to these (tumour) self-antigens has been induced or, in the absence of costimulatory signals, peripheral T cells simply have ignored these antigens or become tolerant ([84] and discussion below on the 'danger' model). In this regard, induction of tumour antigen-specific T cell anergy in adoptively transferred cells has recently been shown in experimental models to be an early event in the course of tumour progression [85]. In addition, studies with transgenic mice that develop spontaneous tumours have shown that vaccination with tumour cells transduced with cytokines fails to inhibit tumour onset and progression, whereas the same cells are able to immunise non-transgenic mice subsequently grafted with tumours [83]. Thus, the failure of naturally established tumours to present antigens efficiently, and to attract and activate tumour-specific T cells at the tumour site, may impede successful vaccination against cancer antigens. Of note, ignorance by the immune system can abort most of the immunotherapy manoeuvres being tested and discussed above. An obvious consequence is that cancer vaccines should be able by design to break down tolerance to tumour antigens.

Immune system deficiencies can, in turn, be either generalised or regional, including in the latter case the active suppression by the tumour of host antigen presentation and of effector cells in the local micro-environment by expression of a variety of molecules. (For reviews on the mechanisms involved in tumour escape see refs. [86, 87].) Clearly, the presence of immunosuppressive factors in tumours suggests the need to complement any immunotherapy strategy with manoeuvres explicitly addressing the intratumoral presence of inhibitors of the immune system response, a combined strategy which to our knowledge is yet to be directly tested. An additional general feature of the immune response to consider when designing gene-based immunotherapy is the redundant phenomenology of the immune system. Its destructive power, occasionally needed in its entire exuberance, requires a complex network of balances and counterbalances to control the pathways of activation and termination of the immune response. Interventions directed to supplement or inhibit single mediators will most probably yield partial physiological and therapeutic results in the best case, may frequently yield no result at all, and occasionally

will produce effects opposed to those desired. Thus, combinations of cytokines are increasingly being used to try to control the complexity of the immune response against tumours. In the field of organ transplantation, successful induction of tolerance to prolong organ survival has been achieved by blocking multiple effector cells and mediators of the adaptive and innate immune systems. Similarly, it is conceivable that breaking the tolerance to tumours will require a strategy of multiple interventions including several target cells and cytokines.

NOVEL STRATEGIES TO OVERCOME CURRENT LIMITATIONS

As we have reviewed above, gene transfer therapies are remarkably successful in *in vitro* and *in vivo* animal model systems. In effect, we already know that the malignant phenotype can be reverted in tumour cell lines by 'knocking-out' or adding certain genes; that tumours can be eradicated by delivery of cytotoxic genes followed by treatment with appropriate pro-drugs; and that tumours can be cured in murine models by making the tumour cells either more immunogenic or by making the immune system cells more responsive, via the expression of cytokines, or by induction of costimulatory and immunogenic molecules. However, overriding limitations have been made apparent in pre-clinical experiments and in the first human gene therapy clinical trials against cancer, as emphasised by the Orkin-Motulsky report to the NIH [88] and the first published clinical results. Most difficulties in obtaining clinically relevant benefits come from the inefficiency of current gene vectors in transducing tumour or immune cells and their inability to access in a selective way target cells distributed systemically. Several avenues for improvement have been proposed, and some will be succinctly reviewed in this section.

Mutation compensation requires quantitative gene transfer

For mutation compensation strategies to work successfully, it seems that every tumour cell would have to be corrected in its genetic defect to achieve a therapeutic outcome. Thus, quantitative transduction of therapeutic genes into the tumour after *in situ* administration of the gene therapy vector may be an essential requirement. To this end, a variety of vector amplification strategies are being explored, including replicative [89, 90] and integrative [91] viral systems.

Replicative vector systems. One method to circumvent suboptimal tumour transduction of therapeutic genes *in vivo* would be the use of conditionally replicative viral vectors: a replication-competent virus would be employed to replicate selectively within infected tumour cells, leaving normal tissues unaffected. Production of progeny virions from the infected tumour cells would then allow infection of neighbouring tumour cells. Thus, the intratumoral viral inoculum would increase, improving the tumour transduction efficiency. In addition, the use of viruses that display a lytic life cycle would allow virus-mediated oncolysis. This effect would occur irrespective of the delivered transgene. In both cases, an amplification of the antitumour effect would be achieved [90, 92]. The limitations of non-replicative vectors already observed in human trials have facilitated rapidly increasing acceptance of this experimental strategy, once considered an eccentric endeavour.

For clinical application of this strategy, a virus with *in vivo* stability and the capacity for conditional replication within tumour cells is mandated [93]. Lack of integration of the viral genome into the cell chromosome seems also desirable. In this regard, both recombinant adenoviruses and herpes viruses have the potential to provide the required properties. Not only do they display high efficiency and stability *in vivo*, but also their replication can be controlled. In the case of adenoviruses, replication can be restricted to tumour cells by placement of genes needed for viral replication under the control of tumour- or tissue-specific transcriptional control elements, such as the promoter of the prostate-specific antigen (PSA) [94]. Alternatively, mutant adenoviruses have been designed to replicate selectively in cells lacking functional *p53*. Because *p53* is absent in many tumours, the replication of this lytic adenovirus would be selective in tumours, and a therapeutic strategy for cancer based on this concept has been proposed [95]. Clinical trials using this virus are currently ongoing, and encouraging preliminary results have been presented [96]. However, extensive studies in a variety of cell lines and animal tumour models have to date failed to confirm the selective properties of the virus to replicate only in *p53* mutant tumour cells [97, 98].

Herpes viruses have also been developed that replicate conditionally in dividing or tumour cells. This selectivity is based on several possible mutations engineered in the viral genome that prevent it from replicating unless the infected cell provides for a substituting molecular activity [99]. These properties have established brain tumours, which are surrounded by non-mitotic cells, as an ideal therapeutic model for testing replication-conditional herpes vectors. Notably, clinical trials have already begun to test both adenovirus and herpes virus-based replicative vector systems for the treatment of human cancer.

A small, non-pathogenic virus called parvovirus went through human trials of viral oncolysis several years ago. The ability of this virus to replicate depends on factors associated with proliferation and differentiation, and as a consequence the virus preferentially displays a cytopathic effect in transformed cells. However, the capacity of the virus to replicate and spread robustly within a solid tumour, and subsequently to induce tumour lysis, appears to be limited.

As another intriguing example, the capacity of human reovirus to replicate selectively in tumour cells having an activated Ras signalling pathway has recently been described in an *in vivo* model [100].

Further refinements in replicative vectors are anticipated that can significantly enhance the possibilities for the realisation of a practical clinical benefit in the context of virus-mediated cancer treatment. A systematic analysis of the life cycle of a replicative virus reveals four areas where further engineering of vectors can bring the required improvements. Thus, better vectors would have increased infectious capacity, would replicate with tight selectivity in target tumours or tissues, would have an enhanced replicative 'burst', and would modulate the local immune response allowing unimpeded regional dissemination throughout the tumour to the required extent. Efforts to realise each of these features have already begun in several laboratories [101–103]. As early examples, our group is developing defective adenoviral vectors that replicate selectively under the stimulus of the cytokine interleukin-6 [104], or under the controlled addition of second vectors carrying replication-enabling DNA sequences [105, 106].

Prolonged transgene expression: integrative vector systems. Lack of stability *in vivo* has confined the use of retroviruses to the *ex vivo* modification of target cells. For *in situ* gene delivery, vectors with high efficiency and stability *in vivo* are needed. Of vector systems with both characteristics, adenoviruses have been most extensively characterised and used (Table 5). However, adenoviruses also have important limitations. In addition to a significant inflammatory and immune response, an additional basis for the limited transgene expression associated with adenoviral vectors derives from their non-integrative nature, such that vector sequences are not retained in the host genome and are not inherited by progeny cells. In this regard, after adenoviral-mediated gene transfer, the recombinant genome is present as an episome in infected cells. Thus, with the proliferation of transduced cells, vector sequences are lost, with the consequence of limited duration of transgene expression. For utility in mutation compensation, and in other gene therapy strategies it thus would be desirable to develop methods to achieve integration of adenoviral vector-delivered transgene sequences in infected cells. As a novel approach to meet this need, we and others have developed a chimeric viral vector system that exploits favourable aspects of both adenoviral and retroviral vectors. In this scheme, adenoviral vectors induce target cells to function as transient retroviral producer cells *in vivo*. The progeny retroviral vector particles can then effectively achieve stable transduction of neighbouring cells [107, 108]. Thus, the principle of combining selected features of available vectors into novel chimeric vectors is being explored in the development of virus-based gene transfer systems [109].

Lentiviruses are retroviruses that, in contrast to other members of the family, can infect both dividing and non-dividing cells. This fundamental feature has driven significant efforts for the development of recombinant lentiviral vectors, although practical issues related to the production and safety have to date limited its widespread use. The recent development of novel vector packaging systems can significantly facilitate availability [110], and new self-inactivating lentiviral vectors can allow safer use [111, 112]. Efficiency of transduction of potential cellular targets by pseudotyped lentiviral vectors and *in vivo* utility are intriguing, and have begun to be described [112, 113].

Prolonged transgene expression: immune tolerance to viral vectors. Gene delivery via adenoviral vectors has been associated *in vivo* with the induction of characteristically intense inflammatory and immunological responses. A number of specific cellular and humoral immune effector mechanisms, together with non-specific innate defence factors, eliminate the infecting virus [114–117]. This process, refined over the course of millennia for maximal efficiency, has been associated with attenuation of expression of the transferred therapeutic gene due, at least in part, to loss of the vector-transduced cells. Based on an understanding of the biology of this phenomenon, specific strategies have been developed to mitigate the process [103]. Of note, the recent development of replicative viral vector systems will mandate the effective modulation of the anti-viral immune response.

Manoeuvres to minimise the immune response against viral vectors include manipulations of both the vector and the host. Firstly, recombinant viral vectors can be genetically engineered to delete viral genes encoding highly immunogenic or cytotoxic viral proteins. However, viral vectors with most of their genomes deleted are more difficult to propagate and purify, transgene expression tends to be unstable, and the vectors are still not totally devoid of immunogenic properties. However, the most recent versions of these vectors may provide adequate production and non-toxic, sustained expression of encoded genes for several months [118]. Alternatively,

Table 5. Gene transfer systems used clinically against cancer

Type	Vector system	Duration of expression	Clinical trials (No.)	Distinguishing features
Nonviral	Liposomes	Transient	30	Receptive and safe administration feasible, inefficient gene delivery, transient expression.
	Naked DNA or RNA (injection, gene gun, electroporation)	Transient	5	Easy preparation, inefficient gene delivery, transient expression.
	Molecular conjugates	Transient	–	Flexible design, inefficient gene delivery, transient expression, unstable *in vivo*.
	Bacteria		–	Useful as vaccines for gene delivery into antigen presenting cells
Viral	Retrovirus	Prolonged	63	Integrates into the chromosome of dividing cells, unstable *in vivo*.
	Adenovirus	Transient	34	Highly efficient *in vivo*, production in high titre, tropism can be modified, induces potent inflammation and immunity, replicative vectors available.
	Poxvirus (vaccinia)	Transient	15	Extensive clinical experience with parent virus, large insert capacity, induces potent inflammation and immunity.
	Adeno-associated virus	Prolonged	–	Non pathogenic, low insert capacity, difficult to scale-up.
	Herpes simplex virus	Transient	1	Highly efficient *in vivo*, large insert capacity, cytotoxic, replicative vectors available.
	Chimeric vectors (e.g. ad/retro)	Prolonged	–	Combine features of component genetic vectors.
	Lentivirus	Prolonged	–	Integrates into the chromosome of *both* dividing and non-dividing cells, well-characterised production system not yet established.

*Registered in the NIH Office of Recombinant DNA Activities in December of 1998 (http://www.nih.gov/od/orda/protocol.pdf).

different serotypes and species of adenoviruses have been proposed to minimise the stimulus for an immune response. Secondly, vectors have been modified to express immunomodulatory molecules. It has been hypothesised that this could create a locally privileged environment for the vector. Some of these engineered molecules are viral genes that interfere with the apparatus of antigen presentation [119], such as the adenoviral glycoprotein 19 K, the herpes simplex virus (HSV) immediate early protein ICP47, or the viral interleukin 10 [120]. Others are recombinant molecules designed to imitate the viral proteins mentioned, such as antisense oligonucleotides or single-chain antibodies against MHC class I and II proteins, or to block costimulation, such as CTL4Ig [103].

Interventions on the immune system of the host have been adopted from common practices in the field of organ transplantation. In this regard, virally transduced cells have been considered to behave, to some extent, as allogeneic cell transplants. Thus, drugs are employed that inhibit the cellular immune response, such as anti-CD4 antibodies, cyclosporine, dexamethasone, and FK 506. In addition, drugs that decrease the humoral immune response, such as cyclophosphamide and deoxyspergualin, have been used. Recently, several groups have demonstrated transient and more specific immune blockade with inhibitors of T cell costimulation, such as anti-CD40 ligand, CTL4Ig, and anti-LFA-1. Furthermore, interventions aimed to decrease the innate response have recently been attempted. For instance, a soluble tumour necrosis factor receptor has been shown to greatly reduce the early adenovirus-induced inflammatory response, and to prolong expression of encoded genes [121]. Unfortunately, the required chronic administration of these immunosuppressive drugs affects systemic immune function and could lead to a number of potential complications, such as infection and malignancy. This makes them less attractive in principle for clinical application, although short-term treatment in cancer patients should be feasible. Lastly, a more specific intervention, induction of tolerance to adenovirus vectors, has been induced by several manoeuvres, including intrathymic injection of adenovirus [122], oral ingestion of adenoviral antigens [123], and infusion of antigen-presenting cells infected with adenovirus and expressing Fas ligand [124]. Thus, although inflammatory and immunological issues have limited the overall utility of adenoviral vectors for gene therapy applications, many of the aforementioned strategies appear promising, and may ultimately allow these limitations to be overcome, at the very least in the context of cancer treatments.

Molecular chemotherapy requires selectivity and amplification

Any approach to cancer gene therapy involving either molecular chemotherapy or mutation compensation requires a high level of efficiency of gene transfer specifically to the tumour cells. Selective gene delivery is necessary because the number of vector particles available for delivery to the cancer cells would be decreased by sequestration by normal, nontarget cells. This would then allow ectopic expression of the delivered therapeutic gene, with possibly deleterious consequences for the normal cells [125].

To date, *in vivo* cancer gene therapy strategies have been restricted to the treatment of compartmentalised tumours in an attempt to achieve high local vector concentrations and relatively efficient tumour transduction. Thus, molecular chemotherapy has been employed in a number of animal models and clinical trials in which adenoviral or retroviral vectors or retroviral vector-producing cells expressing a toxin gene have been directly injected into localised neoplasms confined within body cavities [72, 126–129]. The tumours treated in this manner include glioblastoma, mesothelioma and ovarian carcinomas.

However, these attempts to restrict expression of the therapeutic gene to the target cancer cells merely by confining vector administration have proved inadequate. In this regard, locally administered adenoviral vectors carrying the HSV-*tk* gene have been shown to disseminate, probably as a result of leakage into the blood stream, resulting in a high level of liver-associated toxicity [51]. Substantial hepatic toxicity related to the absence of tumour cell-specific targeting has also been demonstrated in adenovirus-mediated transfer of the HSV-*tk* gene in an ascites model of human breast cancer [72]. In addition, *in situ* injection of adenoviral vectors has been associated with a low level of efficiency of gene transfer to the disease cells in human clinical trials [71]. This phenomenon has been correlated with a paucity of primary receptors on the cancer cells [101, 130]. Hence, it is apparent that there is a need to develop a vector which will achieve a high efficiency of gene transfer selectively to target tumour cells following compartmentalised administration in order to increase the therapeutic index and realise the full potential of gene therapy as a safe approach to the treatment of cancer. Moreover, it is clear that the presently available vectors are inadequate for the treatment of metastatic disease. In order to achieve gene delivery to disseminated cancer cells, the vector must be administered i.v. In this context, there is a stringent demand for specificity of gene delivery to the tumour cells, both in order to avoid vector wastage following transduction of nontarget cells and, more importantly, to prevent toxicity associated with expression of the therapeutic genes in normal cells [125]. Therefore, a means must be developed to modify the gene delivery vehicle to permit efficient gene expression specifically in target cancer cells.

Targeting. Targeted gene therapy for cancer can be accomplished at different levels [131]. In one approach, the tumour cell can be targeted at the level of transduction to achieve the selective delivery of the therapeutic gene. This involves the derivation of a vector that binds selectively to the target cancer cell. Alternatively, the therapeutic gene can be placed under the control of tumour-specific transcriptional regulatory sequences that are activated in tumour cells but not in normal cells and, therefore, target expression selectively to the tumour cell. In addition, targeted cancer gene therapy can exploit the unique physiology of solid tumours.

Transductional targeting. The ability to alter the binding tropism of viral vectors is based on an understanding of the basic biology of viral entry. In this regard, attempts to modify the tropism of adenoviral vectors have been facilitated by the fact that the entry of adenoviruses into susceptible cells requires two sequential steps involving the interaction of two distinct viral capsid proteins with specific receptors on the surface of the target cell. The initial high affinity binding of the adenovirus to the primary cellular receptor (designated the coxsackievirus and adenovirus receptor, CAR [132, 133], occurs via the carboxy-terminal knob domain of the fibre [134, 135]. The next step in infection is internalisation of the virion by receptor-mediated endocytosis potentiated by the interaction of Arg-Gly-Asp (RGD) peptide sequences in the penton base with secondary host cell receptors, integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ [136, 137].

Therefore, strategies to alter adenoviral tropism are based on modification of the viral capsid proteins, fibre and penton base, to permit the recognition of alternative cell-specific receptors. To this end, we have shown that it is possible to redirect adenoviral infection by employing the Fab fragment of a neutralising anti-knob monoclonal antibody (MAb) chemically conjugated to a cell-specific ligand [138–144]. When complexed with preformed adenoviral vector particles, the bispecific conjugate simultaneously ablates endogenous viral tropism and introduces novel tropism, thereby resulting in a truly targeted adenoviral vector. We have employed a number of targeting ligands, including folate, basic fibroblast growth factor, and an antibody directed against the epidermal growth factor receptor. In this manner, we have demonstrated that tropism-modified adenoviral vectors can infect cells that are refractory to transduction by the native vector; that tropism-modified adenoviral vectors can enhance gene transfer to target cells; and that this enhancement in infection can be translated into a therapeutic benefit *in vivo*. Wickham and colleagues have similarly retargeted adenoviral vectors by means of bispecific antibodies, in this case comprising a MAb to an epitope engineered in the penton base and a MAb to a cell surface receptor [145, 146]. However, this approach to the generation of tropism-modified adenoviral vectors suffers from a number of limitations. In particular, since the targeting ligand is not covalently coupled to the adenovirus particle, there is the potential for the bispecific conjugate to become dissociated from the vector.

The drawbacks inherent in any strategy to redirect adenovirus tropism by complexing the vector particles with bispecific targeting conjugates could be avoided by the direct genetic engineering of the viral capsid proteins to contain cell-targeting ligands. In this regard, the carboxy terminus of the adenovirus fibre protein can be modified to incorporate targeting motifs with specificity for cellular receptors [147–150]. In an alternative approach, it has also been reported that targeting ligands can be incorporated within the so-called HI loop of the fibre knob [101, 151]. Adenoviral vectors which have been engineered to incorporate either a polylysine motif at the carboxy terminus of the fibre [147, 150] or an RGD motif at the carboxy terminus [149] or in the HI loop [101] have demonstrated significantly enhanced infection of cancer cell lines and primary tumour cells which express low levels of the primary adenovirus receptor. Thus, these genetic modifications to the fibre protein have resulted in expanded tropism by successfully redirecting adenovirus binding to alternative cellular receptors.

The next challenge in this field will be to employ genetic methods to engineer adenoviral vectors with specificity for a single target cell type. In addition to recognising novel receptors, such vectors should also lack the ability to bind to the native primary adenovirus receptor. This could be accomplished by site-directed mutagenesis of the fibre knob domain to eliminate the cell-binding site. An important consequence of the ablation of native adenovirus tropism is that it will not be possible to propagate these vectors on standard cell lines that express the fibre receptor. However, we have recently developed a novel artificial primary receptor that can be recognised by adenovirus vectors that fail to bind the native fibre receptor [152]. This technology should be useful in the propagation of genetically modified, truly targeted adenoviral vectors.

In contrast to adenoviruses, retroviruses employ a single envelope glycoprotein to accomplish both binding to the cellular receptor and the subsequent step of membrane fusion. As a consequence, modification of retroviral tropism has proven problematic, with few reports of modified envelope proteins which retain these two functions of binding and fusion [153]. A number of molecules, including single-chain antibodies, growth factors and cytokines, can be genetically incorporated into the retroviral envelope glycoprotein, whereupon they confer novel binding specificities into the engineered viral particles. However, some of these surface displayed polypeptides failed to mediate retroviral infection; rather, they proved inhibitory to gene delivery by the modified vectors. In an elegant approach to overcome this obstacle, Russell has incorporated a protease cleavage site into the design of the retargeted vector. Thus, upon contact with proteases expressed on the surface of the target cell, the inhibitory polypeptide is cleaved from the viral surface, thereby restoring infectivity. To date, tropism-modified retroviral vectors have suffered from significantly lower viral titres than the parental vectors and it is therefore not yet proven possible to employ targeted retroviruses *in vivo*.

A key factor in any transductional targeting scheme is the availability of appropriate specific molecules on the target cells that can be exploited. To date, a somewhat restricted range of targeting moieties have been chosen either for proof of principle or for their ability to bind to the relatively short list of previously identified cellular receptors. However, a number of groups have described systems which fundamentally share the similarity of examining libraries of peptides displayed on the surface of bacteriophage for their ability to bind to specific cell types, both *in vitro* and *in vivo* [154, 155]. Thus, a powerful new technology has been developed which allows the rapid isolation and screening of potential tumour-specific ligands, without requiring that the target of the ligand be identified. This approach should, therefore, prove to be a high throughput method to permit the derivation of transductionally targeted vectors for cancer gene therapy.

Transcriptional targeting. Transcriptional targeting has found wide application in the area of molecular chemotherapy where tumour- or tissue-specific regulatory sequences have been employed to restrict expression of the prodrug-converting enzyme specifically to the target cancer cells. For example, transcriptionally targeted adenoviral vectors expressing toxin genes under the control of the tumour-specific alpha-fetoprotein promoter have been employed in molecular chemotherapy approaches to hepatocellular carcinoma [156, 157]. The selective expression of the therapeutic gene in the target hepatomas suggests that transcriptionally targeted adenoviral vectors would be of clinical utility in other diseases. However, it has been reported that certain tumour-specific regulatory elements lose their specificity in the context of an adenoviral vector. Further limitations come from the prohibitively large size of many regulatory sequences, which exceed the capacity of certain current vectors. However, novel gene transfer systems with larger capacity are being developed and could be employed to overcome this limitation—these vectors include gutless adenoviral vectors [158] and reviewed in [117] and recombinant herpes virus [159].

To date, targeted gene therapy has been attempted by employing either transductional targeting or transcriptional targeting alone. However, it should be possible to enhance the overall level of specificity by combining the complementary

approaches of transductional and transcriptional targeting, each of which might be imperfect or 'leaky' by itself [131].

Targeting strategies exploiting tumour physiology. As described above, current approaches to targeted gene therapy for cancer have exploited cellular and molecular differences between normal and malignant cells. However, the physiology of solid tumours at the micro-environmental level provides a unique and selective target for cancer treatment [160, 161]. The regions of hypoxia and necrosis within solid tumours present opportunities for targeted, tumour-selective gene therapy. For example, the hypoxic environment of solid tumours provides a selective means to control gene transcription based on lower oxygen levels compared with normal tissues. Gene therapy strategies activated by hypoxia could include the transcriptional control of a prodrug-activating enzyme by a hypoxia-responsive element. Of course, this approach will still require a means of delivering the constructs specifically to the tumours. Gene therapy strategies could similarly be designed to exploit tumour necrosis. In this regard, certain species of anaerobic bacteria of the genus *Clostridium* can selectively germinate and grow in hypoxic/necrotic regions of solid tumours after i.v. injection of spores [162]. Thus, it might prove possible to exploit clostridia as gene therapy vectors engineered to express therapeutic genes, e.g. a prodrug-activating enzyme.

Modulation of the bystander effect. Limitations of current vectors preclude direct genetic modification of a significant proportion of malignant cells in tumours. It is, therefore, of paramount importance for obtaining clinically relevant results to extend the effects of therapeutic gene expression from the transduced cells to neighbour non-modified cells. Several manoeuvres may be undertaken to extend the magnitude of this required bystander effect. First, survival of genetically modified cells can be prolonged. By doing this, modified cells can sustain longer the expression of the therapeutic gene, thus enhancing the exposure of bystander cells to its protein product. For example, the expression of the cyclin-dependent kinase inhibitor p27 inhibits DNA synthesis and, thus, renders the cells resistant to concomitant herpes simplex virus thymidine kinase/ganciclovir (HSV-*tk*/GCV) treatment. These cells with augmented survival are thus, allowed to prolong the time during which they can pump out cytotoxic metabolites, and hence the bystander effect is increased [163]. However, this intervention should not compromise the capacity for eradication of the genetically modified cells, which could dangerously equate this strategy to the genetic induction of resistance to treatment. Second, the definition of the molecular basis of the bystander effect allows novel interventions to increase directly its magnitude. The intercellular gap junctions, for instance, are known to mediate at least in part the bystander effect of HSV-*tk*/GCV treatment. Retinoic acid and the drugs apigenin and lovastatin upregulate the function of the gap junctions, and have recently been shown to increase considerably the killing effect of HSV-*tk*/GCV both *in vitro* and *in vivo* [164, 165]. Conceivably, genes that encode gap junction molecules can also be transferred into tumour cells for increasing the bystander effect. Third, it is possible to employ therapeutic genes that can be secreted and exert their function in an autocrine and paracrine manner, thus extending regionally their effects against the tumour or its supporting stroma. For instance, the secretion of a soluble receptor for an essential angiogenesis factor can compete regionally for the natural receptor. This blockade limits binding of the angiogenic growth factor to the natural receptor, and consequently restricts the development of the tumour vasculature, thus leading to tumour suppression [23, 24]. There is, therefore, accumulating evidence that the modulation of the bystander effect can regionally amplify the effects of therapeutic gene transfer, and can contribute to overcoming the limitations of current vector systems.

Cellular vehicles. Vectors with the capacity for targeted systemic gene delivery have not been available, and this fact has limited the overall efficacy of gene therapy in cancer, including molecular chemotherapy strategies. As an alternative to viral and other nonviral vectors, cells have been employed for gene delivery. In this approach, the cells are removed from the body and therapeutic genes are transferred into them extracorporally, followed by autologous re-implantation into the patient. In this manner, the genetically modified cell becomes itself the ultimate vector for gene delivery. Examples of primary cells commonly used in this context, so-called 'cellular vehicles', are T lymphocytes, hepatocytes, and fibroblasts.

For application of cell vehicles in the context of disseminated diseases, a cellular vector should possess the attributes of systemic distribution and appropriate tropism, and should be readily available. In this regard, circulating endothelial progenitors have recently been described [166, 167]. Phenotypically, these cells are characterised by the expression of the cellular surface markers CD34 and Flk-1, a receptor for vascular endothelial growth factor. A very intriguing aspect of their behaviour, originally described in animal models of limb ischaemia, is their capacity to localise into areas of angiogenesis after their systemic administration. A variety of genes could conceivably be introduced in these cells, and expression of genetic payloads could be obtained in the environment where these cells ultimately localise. A locoregional effect subsequent to the expression of the therapeutic gene would thus be achieved in areas otherwise poorly accessible to gene transfer. Therefore, endothelial progenitors may represent a novel cellular vector approach with unique features, based on their capacity for systemic circulation and their peculiarly advantageous natural tropism to areas of active angiogenesis. To be exploitable in a gene therapy context, however, it is critical for these endothelial progenitors to be primarily amenable to efficient and safe genetic modification for delivery of the payload therapeutic genes. Unfortunately, genetic modification of human and non-human primate $CD34^+$ cells with a variety of viral vectors has been persistently hampered by very low efficiency. Efforts are currently undergoing in several laboratories, including ours, to improve gene transfer into $CD34^+$ cells without unduly compromising their phenotype and function by using novel vectors *ex vivo*, and for evaluating the potential of endothelial progenitors for systemic gene delivery into metastatic cancer.

In addition to autologous cells, gene therapy based on genetic modification of non-autologous cells has been attempted. Protection within immuno-isolating devices would allow implantation of well-established recombinant cell lines in different hosts, offering a cost-effective approach to gene therapy of cancer when long-term treatment is required [168].

Genetic immunopotentiation to break immune tolerance to tumours

Cancer immunotherapy is yet to be realised as a therapeutic approach in the oncologist's armamentarium. New

ways to consider the immune response against tumours are probably needed if gene transfer is going to be applied in a clinically relevant way. Novel gene therapy approaches that exploit the accumulating knowledge on cytokines and cells involved in the immune response are mounting. They have been reviewed extensively [169, 170]. We would rather first emphasise a novel conceptual framework developed in recent years that can offer new insights on the entire approach of cancer immunotherapy. Secondly, we will focus on gene therapy strategies that, within this theoretical framework, seem particularly apt for offering useful biological information and therapeutic potential.

Danger versus tolerance. The classical paradigm of tumour immunology considers the responses of the immune system to follow a model of discrimination between 'self' and 'non-self' antigens. According to this paradigm, cancers, as microbes, are 'non self' and a major function of the immune system is to seek out and destroy new cancers as they arise. The practical corollary has been a very intense effort to develop tumour 'vaccines'. However, an alternative theoretical model has been proposed by Polly Matzinger to explain and modulate the relationship between the immune system and a genetic disease [171]. In this view, termed the 'danger' model, the need to defend the organism against exogenous lethal pathogens and the need to avoid lethal auto-immunity are equally balanced. According to this new paradigm, to avoid auto-immunity the default reaction of T cells to antigens on non-haematopoietic tissues is tolerance, and it is the role of the antigen-presenting cells to detect and report to T cells situations of dangerous tissue distress (for instance, the beginning of either an inflammatory reaction or tissue damage) that are worth its activation into cytotoxic T cells [84, 172]. If tissue cells normally induce tolerance in susceptible T cells, it is predicted that the default immune response to tumour antigens occurring in those tissues is tolerance as well.

This model can change the emphasis applied in certain immunotherapy strategies. In the classical model, importance is given to the identification of tumour antigens and elaboration of vaccines based on these antigens. Furthermore, it is expected that once activated, the immune response against cells bearing tumour antigens will proceed until their complete elimination. In contrast, the danger model would suggest potentially more relevant new goals such as the orchestration of inflammatory processes in tumour foci, the activation of dendritic cells and other antigen presenting cells, and the direction of T lymphocytes towards the tumour. In other words, the aim should be to recruit not only the adaptive immune response but also and most importantly the cells (macrophages, neutrophils, NK cells) and mediators (cytokines, chemokines) of the innate immune system [84] that establish the immune response in the context of activating 'danger', and make it distinct from tolerogenic immune responses. Importantly, these manoeuvers, including vaccination, should be maintained until elimination of the tumour to avoid its default tolerogenic effects.

Polynucleotide immunisation. Pursuant to the successful application of the strategies of mutation compensation and molecular chemotherapy, obtaining vector targeting and amplification is a critical goal. In contrast, for some genetic immunopotentiation strategies, it may appear that a sophisticated vector is not absolutely needed to facilitate the otherwise inefficient transfer of DNA into tumour or immune system cells.

The possibility exists for eliciting potent, prolonged, and specific immune responses through the intramuscular injection of fragments of nucleic acid encoding tumour-associated antigens [173, 174]. This so-called 'polynucleotide immunisation' (PNI) approach offers several advantages with respect to classic protein immunisation. First, synthesis of the antigen (or antigens) in eukaryotic cells *in vivo* is more likely to result in a protein that is correctly folded and with its antigenic domains adequately presented. Second, PNI elicits a $CD8^+$ cytotoxic T lymphocyte response in addition to a humoral response. Third, long term expression of the encoded antigen may favour long-lived immunity. Of note, the danger model would recommend that, to avoid toleration, repetitive immunisations that involve local inflammatory responses should be administered to keep the association between danger signals and the encoded antigens. Fourth, several nucleotides could easily be combined for induction of responses against multiple relevant antigens. Fifth, safety concerns related to virus-derived or cell derived vaccines are obviated. Sixth, manufacturing and use of recombinant DNA may have economical and logistic advantages with respect to standard vaccines. Polynucleotides in the form of both DNA and RNA can be used. For example, plasmid DNA encoding carcinoembryonic antigen, a non-transforming tumour-associated antigen, has shown prolonged humoral and lympho-proliferative responses in non-human primates [174], and is being tested in a clinical protocol for colorectal cancer patients. Transforming tumour-associated antigens, such as erbB-2, may be encoded by RNA constructs that avoid the risk of integration of a potentially oncogenic sequence and are expressed only transiently. Once the antigen is expressed in myofibres, its presentation to the effector cells follows an unknown pathway, but is known to induce antibody production, T cell proliferation, lymphokine release, generation of CTL, and delayed hypersensitivity reactions. Importantly, encouraging results in animal models have been followed by clinical trials for both immune protection and therapeutic applications. Although tumours are antigenically heterogeneous, the hypothesis is that immune responses against the polynucleotide-encoded antigens can break immune tolerance for the tumour via a single epitope, which, in turn, would alert the immune system to the existence of the tumour as a foreign entity, provoking a systemic response.

Enhanced antigen presentation by genetically modified dendritic cells. As we reviewed above, most tumours are ignored by the immune system. Thus, tumour antigen-specific T lymphocytes, which are certainly present in the immune repertoire, are not activated and migrate systemically without showing any special tropism towards its cognate antigens present in the tumour sites. This has been partly attributed to a lack of activation and antigen presentation by dendritic cells (DCs) in tumours [175, 176]. Indeed, DCs infiltrating several tumours lack B7-1 and B7-2 molecules, which reveals a non-stimulatory status and impedes the encounter by T lymphocytes of the required 'signal 2' on DCs for antigen-specific activation. However, when autologous DCs are expanded and exposed *ex vivo* to tumour antigens and these DCs are then reinfused, activation of tumour-specific cytotoxic T lymphocytes ensues. In animal models, this intervention achieves a protective effect against subsequent exposure to tumours and also can induce a therapeutic effect in tumours already present [175]. This strategy is currently being explored in patients [177].

Multiple vectors are being tested for delivering tumour antigens into DCs, including viral vectors, naked DNA, RNA, tumour lysates, and peptides [178, 179]. It is possible that methods that maximise exposure of DCs to a variety of tumour antigens may have an advantage by overcoming the expected emergence of antigen-loss variants as well as natural immunovariation of tumours [180]. Importantly, fusion of DCs and tumour cells have also shown the capacity to revert established immune tolerance [181]. This concept has been tested in transgenic animals tolerant to the antigen MUC1, and refractory to vaccination with irradiated MUC-1 positive cells. Immunisation with the dendritic cell fusion that express MUC1 resulted in the rejection of established metastases and there was no apparent autoimmunity against normal tissues. These findings demonstrate that tolerance to tumour-associated antigens can be reversed, and suggest that immunisation with hybrids of dendritic and carcinoma cells may be a powerful methodology for whole cell vaccination against cancer.

Reversal of tumour-induced immunosuppression. DCs are conceived as a powerful way to stimulate tumour-specific T cells. It may be, however, that a robust generation of such cytotoxic T cells is not enough for rejecting established tumours, as has been shown in elegant animal models [182]. Indeed, this can be predicted from the anatomy of the T cell response, whereby it is critical not only for DCs to uptake tumour antigens in the tumour site, mature and migrate to lymph nodes, and present antigens to T lymphocytes. But it is additionally needed for stimulated antigen-specific T lymphocytes to home assertively into the possibly widespread tumour sites, and keep their activation and proliferation therein, despite numerous immunosuppressive signals. Further strategies may be needed, therefore, for (1) attracting and activating the tumour-specific T cells into various tumour sites [183, 184]; (2) inhibiting the local production of immunosuppressive molecules, such as TGF beta [185], interleukin-10, VEGF, and fas ligand; and (3) counteracting the antigen variation and down-regulation of antigen presentation. It can be concluded that a systematic intervention with multiple targets at the different pathophysiological levels mentioned seems a reasonable programme to achieve a meaningful antitumour immune response.

CONCLUSION

The delineation of the molecular basis of cancer allows the possibility of specific intervention at the molecular level for therapeutic purposes. To this end, three main approaches have been developed: mutation compensation, molecular chemotherapy, and genetic immunopotentiation. For each of these conceptual approaches, human clinical protocols have entered testing in phases I and II to assess dose escalation, safety and toxicity issues, and more recently to evaluate efficacy, respectively. However, major problems remain to be solved before these approaches can become effective and common place strategies for cancer. Principle among these is the basic ability to deliver therapeutic genes quantitatively, and specifically, not only into tumour cells but also into tumour-supporting tissues and effector cells of the immune system. As vector technology fulfils these stringent requirements, it is anticipated that the promising results already observed in pre-clinical studies will translate quickly into the clinic for amelioration of life-threatening malignant diseases.

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REVIEW

Gene Therapy Progress and Prospects: cancer gene therapy using tumour suppressor genes

IA McNeish, SJ Bell and NR Lemoine
Cancer Research UK Molecular Oncology Unit, Imperial College School of Medicine, Hammersmith Hospital, London, UK

Targeting tumour suppressor gene pathways is an attractive therapeutic strategy in cancer. Since the first clinical trial took place in 1996, at least 20 other trials have investigated the possibility of restoring p53 function, either alone or in combination with chemotherapy, but with limited success. Other recent clinical trials have sought to harness abnormalities in the p53 pathway to permit tumour-selective replication of adenoviral vectors such as dl1520 (Onyx-015). Other tumour suppressor genes, such as retinoblastoma (Rb) and PTEN (phosphatase, tensin homologue, deleted on chromosome 10), are the targets for imminent clinical trials, while microarray technologies are revealing multiple new genes that are potential targets for future gene therapy.
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Keywords: *tumour suppressor gene; p53; Rb; PTEN; cinical trial*

In brief

Progress

- Clinical trials of p53 gene replacement have had limited success
- Replicating adenoviral vectors targeting abnormal p53 function have also had limited success in clinical trials
- *Targeting the Rb pathway*: Rb mutants may be more potent tumour suppressors than wild-type Rb
- New oncolytic adenoviruses also target the Rb pathway
- The INK4/ARF locus provides two potential targets for gene therapy
- PTEN expression alters metastatic potential and reduces neovascularization
- Multiple new tumour suppressor genes offer new therapeutic possibilities, especially mda-7 and OPCML

Prospects

- The ability to induce growth arrest and apoptosis *in vitro* does not guarantee clinical success.
- Fuller understanding of downstream targets of p53 and Rb is necessary.
- Clinical trials of second-generation oncolytic viruses targeting Rb pathway will be eagerly awaited
- Combinations of tumour suppressor genes may offer new greater therapeutic potential
- New tumour suppressor genes will be discovered

Introduction

It has long been recognized that the development of invasive malignancy requires multiple genetic events, and modern technologies now suggest that tens, if not hundreds, of genes may be aberrantly expressed in malignant cells.[1,2] In the last decade, studies on p53 replacement have dominated the literature and it remains the only tumour suppressor gene to be evaluated formally in clinical trials. Here, we review the progress that has been made in the past 2 years in the field of tumour suppressor gene therapy and the future prospects for utilizing pathways other than p53, including the well characterized, such as retinoblastoma (Rb) and PTEN (phosphatase, tensin homologue, deleted on chromosome 10), as well as those described more recently, such as melanoma differentiation associated gene-7 (mda-7) and opioid binding protein/cell adhesion molecule-like gene (OPCML).

Correspondence: Dr IA McNeish, Cancer Research UK Molecular Oncology Unit, MRC Cyclotron Building, Hammersmith Hospital, London W12 0HS, UK
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Clinical trials of p53 gene replacement have had limited success

After the promise of the first clinical trial of p53 gene replacement in non-small-cell lung carcinoma in 1996,

those published in the past 2 years[3–7] have been somewhat disappointing. In a neoadjuvant bladder carcinoma trial[5], 12 patients received either intratumoral or intravesical injections of an adenovirus-encoding wild-type p53 (Ad p53) 3 days prior to radical cystectomy. Interestingly, transfection efficiency was much greater following intravesical administration and, overall, 7/11 (64%) evaluable patients (including 7/9 of the intravesical cohort) had evidence of transgene expression by vector-specific reverse-transcriptase PCR (RT-PCR), as well as some evidence of increased expression (both mRNA and protein) of $p21^{Waf1/Cip1}$, a p53 target gene. By contrast, in patients with locally advanced bladder cancer treated with intravesical Ad p53 at comparable doses,[6] only 2/7 (29%) tumours demonstrated p53 transgene expression, with no detectable changes in the expression of either $p21^{Waf1/Cip1}$ or Bax. When comparing transgene expression in these two bladder cancer trials, it is possible that the larger instillation volume (120 ml) and the use of a transfection-enhancing agent in the neoadjuvant trial 5 contributed to the higher transfection rates.

In a phase I recurrent glioma trial, 12 patients received intratumoral Ad p53 at doses between 3×10^{10} to 3×10^{12} particles, followed by tumour resection, at which time more Ad p53 was injected into the tumour bed.[7] Before Ad p53 injection, only one of eight assessed tumours was p53 positive (by immunohistochemistry), while 10/12 showed nuclear p53 staining after injection and 7/8 showed positive staining for $p21^{Waf1/Cip1}$. However, the zone of transfected cells extended no more than 8 mm from the injection site and the median overall survival for the whole cohort was only 43 weeks.

In non-small-cell lung cancer, intratumoral injection of 7.5×10^{12} particles of Ad p53 every 21 or 28 days produced transgene expression in 17/25 (68%) tumours. Patients also received chemotherapy (either carboplatin and paclitaxel or cisplatin and vinorelbine), but the frequency of overall tumour response was the same in Ad p53-injected lesions and noninjected lesions (52 *versus* 48%, respectively). However, there was a suggestion that the Ad p53-treated lesions reduced in size by a greater amount than the noninjected controls.

Ovarian cancer is traditionally thought to be an appealing target for clinical gene therapy because the disease tends to remain localized within the abdominal cavity, so that intraperitoneal vector delivery is a rational strategy. The extensive experience of p53 gene therapy in this disease culminated in a randomized phase III trial in which women with p53-null or p53 mutant tumours were randomized to chemotherapy alone or chemotherapy plus intraperitoneal Ad p53 following optimum debulking primary surgery. However, the first interim analysis indicated that not only did Ad p53 fail to improve effectiveness but was also associated with increased toxicity. As a result, the study has been abandoned (reported in Zeimet and Marth[8]).

Despite the limited clinical efficacy, some positive factors have emerged from these trials. Firstly, it is noticeable that the trials have been designed with credible scientific as well as clinical end points. Secondly, except for the experience in the ovarian phase III trial, of which few details are available, treatment has largely been well tolerated with minimal toxicity. However, one must address why the trials were relatively unsuccessful and two broad possibilities emerge. Firstly, there remains the perennial problem of optimizing gene transfer. Improving gene transfer in the clinical setting with delivery of vectors to tumours disseminated throughout the body is a huge problem and lies outside the scope of this review. Secondly, there remains the possibility that p53 is the 'wrong' transgene. Although p53 mutations are found in many malignancies and defective p53 function may be causally linked to chemotherapy resistance,[9] many aspects of p53 biology remain unanswered, especially what determines whether cells undergo apoptosis or cell cycle arrest in response to p53 activation.[10] There is some evidence that low-level p53 expression, such as is likely to result from adenoviral gene transfer, causes cell cycle arrest rather than cell death. Also, the proapoptotic function of p53 depends upon transactivation of genes such as Bax, Apaf-1, Fas and PTEN, whose own expression or activity may be abnormal in tumour cells.[11] It is known that mutant p53 can act in a dominant-negative manner in p53 tetramers,[12] which could negate the effect of ectopically expressed wild-type protein. Finally, there is evidence that polymorphisms of the p53 gene (especially codon 72 – arginine *versus* proline) can determine the responsiveness of tumours to chemo- and radiotherapy by influencing inhibition of p73.[13] Only once all these issues have been addressed is there likely to be any advance in the field of p53 gene replacement.

Replicating adenoviral vectors targeting abnormal p53 function have also had limited success in clinical trials

The adenovirus E1B 55 kDa protein suppresses p53 function in infected cells and E1B 55K-deleted adenoviral vectors may be able to replicate within and cause cytolysis of tumours with defective p53 function. In the past 2 years, six separate phase I/II trials of such a virus (variously known as *dl*1520, Onyx-015 and CI-1042) have been published, in a range of tumour types, including colorectal,[14,15] ovarian [16] and pancreatic carcinomas,[17,18] and in patients with liver metastases from gastrointestinal malignancies.[19] A total of 93 patients received doses of up to 2×10^{12} viral particles per injection with no objective clinical responses seen in any patient treated with *dl*1520 as a single agent. However, in combination with chemotherapy, some responses were seen; with 5-FU, eight patients with colorectal liver metastases demonstrated either partial or minor responses, at least five of whom had previously been refractory to 5-FU.[14,19] In primary pancreatic carcinoma, two patients had partial responses in combination with gemcitabine.[17]

One complexity in analysing these results is that it is now apparent that cellular p53 status is not the only determinant of the replication of this virus.[20] There have been many reports of replication within cells that are p53 wild type and there is contradictory evidence on the possible importance of the mdm-2/hdm-2 inhibitor $p14^{Arf}$.[21,22] Similarly, E1B 55K almost certainly has functions in addition to p53 suppression, including modulating viral and cellular mRNA nuclear transport and stimulating late viral mRNA translation. Given this, the results of the trials and the uncertainties over p53

replacement, it seems unlikely that any further significant progress will be made with *dl*1520.

Targeting the Rb pathway: Rb mutants may be more potent tumour suppressors than wild-type Rb

Rb is the paradigmatic tumour suppressor gene, originally postulated in 1971. It is the target for transforming viral proteins such as HPV E7 and adenovirus E1A, inactivation of the Rb and p53 genes alone can induce malignancy in mouse models[23] and abnormalities in the Rb pathway and the G1/S checkpoint probably exist in all malignancies.[24] The pathway has many components that are potential targets for therapy (see Figure 1). Upon growth stimulation, cyclin D expression increases and it forms complexes with cyclin-dependent kinase 4 (cdk4) or cdk6 and these complexes sequester the cdk inhibitor $p27^{Kip1}$ from cyclin E/cdk2. The cyclin D/cdk4 and cyclin E/cdk2 complexes are now able to phosphorylate Rb and this phosphorylated form of Rb can no longer bind the E2F family of transcription factors, freeing E2F to transactivate the genes necessary for S-phase entry. The activity of cyclin D/cdk is also controlled by the INK4 family of inhibitors, of which $p16^{INK4A}$ is perhaps the best known.

Rather surprisingly, there have been many fewer studies on replacement of Rb family members than p53. Early reports suggested that the ability of Rb expression alone to inhibit tumour cell growth is variable and Rb expression may, paradoxically, inhibit p53-induced apoptosis.[25] Rb phosphorylation mutants and truncated variants may have enhanced tumour suppressor function compared to the wild-type protein. One such derivative is Rb^{94}, in which translation is initiated from a second AUG codon in the Rb mRNA and which lacks the N-terminal 112 amino acids of the full-length protein. There is evidence that Rb^{94} has a longer half-life than Rb itself and remains in the hypophosphorylated form for extended periods. Two recent reports suggest that adenovirus-mediated Rb^{94} gene transfer can induce apoptosis in models of head and neck[26] and bladder[27] cancers, with minimal effects on nonimmortalized normal cells. Of note, Rb^{94} appears able to induce cell death regardless of the Rb status of tumours, unlike the full-length protein, which is not effective in tumours bearing wild-type Rb. One potential explanation for this is that Rb^{94}, in addition to generating caspase-mediated apoptosis, appeared to induce cell cycle blockade at G2/M (rather than G1) and also rapid telomere erosion with ensuing chromosomal instability. Although it had previously been reported that full-length Rb could inhibit telomerase, the cell cycle findings are novel and as yet unexplained.

Another Rb variant, Rb^{56}, is also a C-terminal derivative. It contains the regions necessary for E2F binding and may be capable of inhibiting E2F-mediated transcription more efficiently than full-length Rb. Recent work on Rb^{56} has demonstrated the ability of a fusion protein, consisting of Rb^{56} and the DP-1 binding domains of E2F to induce cell cycle arrest in vascular smooth muscle cells and inhibit smooth muscle cell hyperplasia in response to intimal injury.[28] Taken together, these reports suggest that Rb mutants and splice variants may be more potent tumour suppressors than Rb. Data on the potential of the other two members of the Rb family, p107 and p130, in gene therapy are very limited, but retrovirus-mediated transfer of the p130 gene can suppress the growth of lung carcinoma cells *in vitro* and *in vivo*.[29]

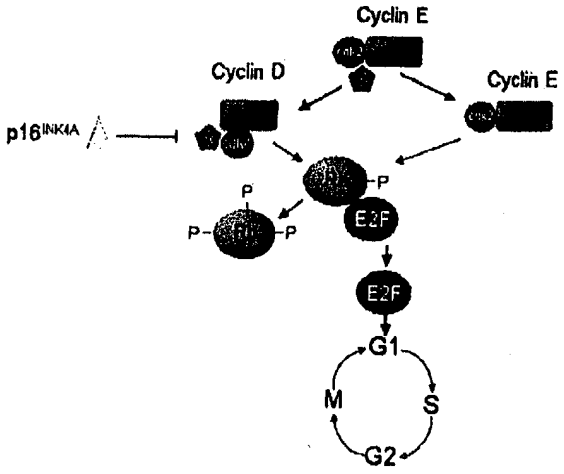

Figure 1 Rb pathway. In response to a mitogenic stimulus, cyclin D/cdk4 complexes form and sequester p27 and other Waf1/Cip1 family members. The cyclin D/cdk4 and cyclin E/cdk2 complexes are then free to phosphorylate Rb. This frees members of the E2F family to transactivate genes necessary for S-phase entry. $p16^{INK4A}$ inhibits cyclin D/cdk4 and thus prevents Rb phosphorylation.

New oncolytic adenoviruses also target the Rb pathway

Following on from *dl*1520, a second generation of selectively replicating adenoviral vectors has now been developed. The viruses nearest to clinical trial specifically target Rb function. The adenoviral E1A protein contains two conserved regions, CR-1 (amino acids 30–60) and CR-2 (amino acids 120–127), the latter critical for binding to and inactivating Rb and whose deletion prevents formation of E1A/Rb complexes. Two similar mutants have been described recently; *dl*922/947 is deleted in amino acids 122–129,[30] while Δ24 is deleted in amino acids 121–128.[31] Both have been assessed in *in vitro* and *in vivo* models of cancer and *dl*922/947 is capable of replicating with much greater efficiency within a panel of tumour cell lines than *dl*1520, with minimal S-phase induction in quiescent nonimmortalized cells.[30] Most recently, Δ24 has been modified further to include a RGD-4C peptide into the adenoviral fibre, which permits infection of cells independent of the normal coxsackie adenovirus receptor that is frequently expressed at very low levels on tumour cells.[32] Δ24-RGD is capable of lysing ovarian carcinoma and glioma cells *in vitro*, as well as extending the survival of mice-bearing xenografts of both tumour types.[32,33] Of note, Δ24-RGD appeared to have a significantly greater cytopathic effect than Δ24 and its replication on normal human astrocytes was at least 3 log scales lower than a wild-type adenovirus.[33] Clinical trials of both Δ24-RGD and *dl*922/947 are imminent.

Further adenoviral mutants also explore targeting of the Rb pathway. Ar6pAE2fF[34] and Onyx-411[35] both have an E2F promoter in place of the adenoviral E1A promoter. In addition, Onyx-411 has a second E2F promoter to drive the expression of the E4 region and is also deleted in the E1A-CR-2 region, like *dl*922/947. The rationale behind these modifications is that the E2F promoter is selectively activated in the presence of a defective Rb pathway and E4 gene products, especially E4 orf4/6, cooperate with E1A and E1B proteins to create a cellular environment that permits efficient expression of viral genes and thus productive viral infection.[36] Both Ar6pAE2fF and Onyx-411 demonstrate tumour-specific replication with minimal effect upon normal cells, including proliferating epithelial cells, and both were more potent and tumour selective than *dl*1520.

INK4/ARF locus provides two potential targets for gene therapy

The INK4/ARF locus on chromosome 9 encodes two separate tumour suppressor genes from alternative reading frames, $p16^{INK4A}$ and $p14^{Arf}$ (also known as $p19^{Arf}$ in mice), which serve to highlight the close link between the p53 and Rb pathways.[24] $p16^{INK4A}$ is a potent inhibitor of the cyclin D/cdk4 complex that phosphorylates Rb, while $p14^{Arf}$ inhibits hdm-2 (mdm-2 in mice), whose functions are to prevent p53-mediated transcription and to promote p53 ubiquitination. Homozygous deletions of the INK4/ARF locus are seen in many malignancies especially melanoma.[37]

Adenoviral delivery of the $p16^{INK4A}$ gene is able to induce cell cycle arrest *in vitro* and, in cooperation with adenoviral p53 expression, induce apoptosis and inhibit tumour growth *in vivo*. It appears that $p16^{INK4A}$ may be a more effective inducer of apoptosis than other members of the INK4 family ($p15^{INK4B}$, $p18^{INK4C}$) or the Waf1/Cip1 family (p21, p27). Interestingly, it appears that $p16^{INK4A}$ is able to induce apoptosis in cells lacking Rb, suggesting that it may have alternative functions. This has been reiterated by more recent work in which the effectiveness of $p16^{INK4A}$ and p53 gene delivery was compared in ovarian carcinoma models with varied $p16^{INK4A}$ and p53 status (wild type, null and mutant).[38] In all cell lines, $p16^{INK4A}$ appears as a more efficient inducer of growth arrest, but not apoptosis, than p53. *In vivo*, however, adenoviral $p16^{INK4A}$ (Ad p16) produces statistically greater survival in $p16^{INK4A}$- and p53-null or wild-type models than Ad p53 alone or even Ad p16 and Ad p53 combined. Clearly, as has been mentioned above, the limited efficacy of Ad p53 could result from abnormalities in downstream effectors of p53-mediated apoptosis. However, the same may be true of pathways downstream of $p16^{INK4A}$. Therefore, it remains possible that $p16^{INK4A}$ has additional functions, of which downregulation of vascular endothelial growth factor (VEGF) is one possibility. Other groups have recently demonstrated the efficacy of adenoviral $p16^{INK4A}$ delivery in lymphoma[39] and glioma[40] models, but one note of caution is necessary. There is evidence that ectopic overexpression of $p16^{INK4A}$ can produce resistance to some chemotherapy drugs, possibly by inducing G1 cell cycle arrest, as many chemotherapy drugs are at their most effect in S phase.[41]

In the past 2 years, more interest has focused on $p14^{Arf}$. Several reports have demonstrated that adenoviral delivery of the $p14^{Arf}$ gene is capable of inducing cell cycle arrest and apoptosis in a wide variety of tumour models[42–47] and can sensitize cells to chemotherapy.[48] Initial reports suggested that intact p53 pathways were required for $p14^{Arf}$-mediated cytotoxicity[47–49] and that cotransfection with wild-type p53 could enhance the $p14^{Arf}$ effect.[46] It now appears that $p14^{Arf}$ is capable of affecting proteins other than p53, such as E2F-1,[50] $HIF1\alpha$[51] and topoisomerase I.[52] Cluster analysis of gene expression patterns in mouse embryo fibroblasts indicates that $p19^{ARF}$ induces expression of both p53-dependent and -independent genes, the latter including members of the B-cell translocation family (Btg/Tob) that can inhibit proliferation in cells regardless of p53 status.[43] Recently, $p14^{Arf}$ has shown itself capable of inducing apoptosis in p53- and Bax-null DU145 prostate carcinoma cells.[42] In p53-null H358 lung carcinoma cells, $p14^{Arf}$ induces arrest in G2 phase, followed by apoptosis. This G2 arrest correlates with inhibition of CDC2, inactivation of CDC25C and induction of $p21^{Waf1}$. Of note, $p14^{Arf}$ is capable of inducing tumour regression in H358 xenografts.[53] One possible explanation for the discrepancy between these results and earlier studies that suggested p53 was an absolute requirement for $p14^{Arf}$-mediated cell death is timing.[49] In p53-null cells, it takes up to 6 days for G2 arrest to take place, in contrast to only 24–48 h in p53-positive cells.[53]

PTEN expression alters metastatic potential and reduces neovascularization

PTEN, also known as MMAC1 and TEP-1, is a phosphatase whose importance as a tumour suppressor gene is being increasingly recognized. Although PTEN can dephosphorylate proteins such as focal adhesion kinase, its primary function is to degrade the products of phosphatidylinositol 3′-kinase (PI-3kinase) by dephosphorylating phosphatidylinositol 3,4,5-trisphosphate and phosphatidylinositol 3,4,-bisphosphate at the 3′ position. One of the main downstream targets of the PI-3kinase pathway is the kinase Akt, also known as PKB (protein kinase B), which, in turn, can activate a wide range of signals that lead to cell proliferation and decreased apoptosis (see Figure 2). Thus, the loss of PTEN activity, which is seen in up to 40% of all malignancies,[54,55] can have diverse effects on cell growth and differentiation.

In the past 2 years, there have been a number of studies investigating PTEN gene replacement, many of which have focused on prostate cancer. In PTEN-null prostate cancer, expression of PTEN causes a decrease in Bcl-2 expression and sensitizes cells to doxorubicin and vincristine chemotherapy,[56] and also sensitizes cells to death receptor-mediated apoptosis that could be overcome with Bcl-2 overexpression.[57] In another prostate model, adenoviral PTEN (Ad PTEN) delivery to PC3 cells *in vitro* leads to G1 arrest, but not apoptosis.[58] Interestingly, when the PC3 cells are transfected with Ad PTEN and then implanted orthotopically into mice, there is no reduction in tumorigenicity, but a significant reduction in the development of lymph node metastases, implying that PTEN may not be a critical regulator

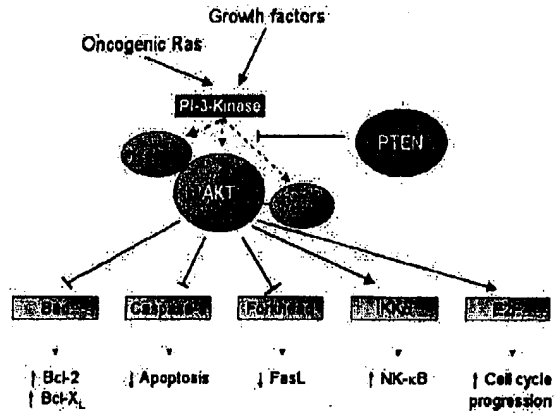

Figure 2 The Akt/PTEN pathway. Oncogenic and mitogenic stimuli that activate PI3kinase can lead to Akt activation, either directly, via the actions of phosphatidylinositol 3,4,5-trisphosphate (PIP_3) and phosphatidylinositol 3,4,-bisphosphate ($PI(3,4)P_2$) on the plectrin homology (PH domain), or indirectly, via 3'-phosphoinositide-dependent kinase 1 (PDK1) and PDK2-mediated phosphorylation at positions T308 and S473. Activated Akt can then modulate multiple cellular pathways, leading to the inhibition of apoptosis and stimulation of cell growth. PTEN has intrinsic lipid phosphatase activity that removes the phosphate moeity from the 3' position of PIP_3 and $PI(3,4)P_2$, and thus counters the antiapoptotic and growth stimulatory activities of PI3kinase and Akt.

of tumour formation and growth, but a controller of dissemination. When Ad PTEN is injected directly into pre-existing prostate xenografts, there is no tumour regression, which further underlines this point.

By contrast, injection of Ad PTEN into bladder xenografts produced demonstrable tumour regression and induction of apoptosis, but only in PTEN-null UM-UC-3 tumours. In tumours that are PTEN wild type, Ad PTEN injection produced only transient growth inhibition.[59] Alongside reduction in phosphorylated Akt expression, another observation from the UM-UC-3 tumours is a reduction in VEGF expression both *in vitro* and *in vivo*, the latter accompanied by a reduction in tumour vessel formation. VEGF is known to be an Akt/PTEN target,[60] and neovascularization is a marker of transformation from low- to high-grade gliomas in humans with PTEN mutations seen almost exclusively in high-grade tumours. Further indication of the potential of PTEN to influence angiogenesis in glioma is shown with U87MG xenografts in mice. In the presence of PTEN expression, *in vivo* growth is reduced, with marked reduction in angiogenic activity.[61] Even in the presence of proangiogenic signals such as constitutive EGFR activation and/or p53 inactivation, Ad PTEN delivery to glioma xenografts in mice produces a marked reduction in tumour vascularity.[62] Therefore, there may be a differential role for PTEN in different tumour types, reducing invasion and metastatic potential in some models and inhibiting tumour vascularization in others.

Multiple new tumour suppressor genes offer new therapeutic possibilities, especially mda-7 and OPCML

Mda-7 (also known as IL-24) is a member of the IL-10 family of cytokines and was first described as a potential tumour suppressor gene, when shown to be expressed on differentiated melanocytes but not melanoma cells. Subsequently, it was shown that adenoviral delivery of the mda-7 gene (Ad mda-7) is able to induce apoptosis in malignant cells but not normal epithelial cells in both melanoma[63] and NSCLC.[64]

Work in the past 2 years has extended knowledge on this gene. Expression is downregulated in a wide variety of malignancies,[65] while restoration of expression via Ad mda-7 can also induce growth arrest *in vivo*.[66] The mechanisms via which mda-7 induces growth arrest and apoptosis are complex. It appears to upregulate the expression of TRAIL and its receptors DR4/5, which could sensitize tumour cells to death receptor-mediated apoptosis.[66] There is also evidence that mda-7 can increase the expression of the RNA-dependent protein kinase PKR in some NSCLC cells.[67] The normal role of PKR is to limit viral infection by inhibiting protein synthesis and hence block viral protein production, but it may also function as a regulator of tumorigenesis. Recently, microarray analysis suggests that Ad mda-7 transfection can alter expression of members of both the β-catenin and PI3kinase signalling pathways in some breast and NSCLC cell lines.[68] Curiously, this analysis was performed on the same NSCLC line (H1299) as had been studied previously,[67] but PKR was not one of the genes whose expression was upregulated. Finally, several reports suggest that mda-7 may have a role in angiogenesis. Ad mda-7 is able to inhibit endothelial cell differentiation and reduce tumour vascularity in human lung cancer xenografts in mice,[66] and purified mda-7 protein is capable of inhibiting endothelial cell differentiation and migration more effectively than endostatin.[69]

Finally, another potential tumour suppressor gene has been identified in ovarian cancer that may have therapeutic potential. OPCML is a member of the family of Ig domain-containing glycosylphosphatidylinositol-anchored cell adhesion molecules and its expression is completely absent in over 80% of ovarian carcinomas, including both established cell lines and primary tumours.[70] Interestingly, the downregulation appears due mainly to CpG island methylation, and restoration of OPCML expression was able to impair ovarian carcinoma cell growth both *in vitro* and *in vivo*.[70] Clearly, more work will be required to evaluate the pathways via which OPCML functions in ovarian carcinoma.

Conclusions and prospects

Although targeting tumour suppressor gene pathways is an attractive and logical strategy for cancer gene therapy, results from clinical trials have not mirrored the preclinical studies. Clearly, the ability to induce cell cycle arrest and apoptosis *in vitro* or growth arrest in mouse xenografts does not guarantee responses in clinical trials. Several specific hurdles must be overcome if such therapies are to become routine. Firstly, a greater understanding of the biology of the ubiquitous p53 and Rb tumour suppressor genes pathways is vital, especially an understanding of their own downstream targets and how these may be altered in malignancy. Secondly, other pathways need to be thoroughly evaluated, especially those that appear to be tumour-type specific.

Surprisingly, little gene therapy work has been published on restoring well-known tumour suppressor genes such as BRCA1 in breast cancer and APC in colon cancer. The novel genes, OPCML and mda-7, may offer new disease-specific pathways to target in ovarian and melanoma/lung carcinoma, respectively. Thirdly, restoring tumour suppressor gene function alone may be insufficient and combination treatments, either with multiple genes (eg one disease-specific and one ubiquitous gene) or a tumour suppressor gene with an apoptosis inducer such as chemotherapy or activated caspases, may be required. However, one thing is certain: extending our knowledge of tumour suppressor genes and their normal roles must ultimately lead to improved therapies for all malignancies.

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Review

Adenoviral vectors: Systemic delivery and tumor targeting

Nicola K Green and Leonard W Seymour

Department of Clinical Pharmacology, University of Oxford, Radcliffe Infirmary, Oxford, UK.

The development of a targeted adenoviral vector, which can be delivered systemically, is one of the major challenges facing cancer gene therapy. The virus is readily cleared from the bloodstream, can be neutralised by pre-existing antibodies, and has a permissive cellular tropism. Clinical studies using the ONYX virus have shown limited efficacy, but there are several hurdles to overcome to achieve an effective tumor-specific systemic therapy. In this review, we have summarized the various strategies used to overcome the limitations of adenoviral-mediated gene delivery.
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The majority of cancer patients present with advanced disease. Often, the primary tumor mass can be resected, although surgery usually cannot be used to remove disseminated metastases that are frequently responsible for ultimate patient demise. Agents capable of treating such disseminated disease must have a high therapeutic index, showing selective toxicity against cancer tissue while sparing normal cells. Gene therapy is one very promising approach because targeting to cancer cells can be combined with intracellular mechanisms regulating selective expression to achieve good tumor specificity. Tumor-selective activity can be used to regulate very great toxicity, either through the amplification intrinsic in producing a gene product from even a single gene copy, or by using a conditionally replicating system to kill infected cells.

Adenovirus is one of the most promising vectors for cancer gene therapy, and the development of a form that can be administered systemically and targeted to disseminated tumors is one of the major challenges facing cancer gene therapy. However, there are several issues to be addressed before this is likely to be a feasible approach. Most obvious is the problem of rapid clearance of virus from the blood, widely reported and discussed by several authors who have used a range of techniques to prevent unwanted entry of the virus into nontarget tissues. These approaches are discussed in detail below. Second is the question of target selectivity, where effective expression in target tissue must be combined with avoiding unwanted infection of healthy tissues in order to prevent unnecessary immune provocation and vector depletion. More fundamentally, however, are the technical issues over which assays can be meaningfully used to measure pharmacokinetics of viruses, particularly when they have been tropism ablated or retargeted to infect through novel receptors.

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Address correspondence and reprint requests to: Dr Nicola K Green, Department of Clinical Pharmacology, University of Oxford, Radcliffe Infirmary, Woodstock Road, Oxford OX2 6HE, UK.
E-mail: nicky.green@clinpharm.ox.ac.uk

Techniques for the determination of adenovirus distribution kinetics

Useful performance of pharmacokinetic analyses requires adequate definition of the virus preparation being used, and understanding of the species being followed when measurements are taken. Typical adenovirus preparations have particle-to-infectivity (P/I) ratios in the range of 10–100. The fact that P/I ratios are usually greater than 1 partially results from the practical design of experiments used to determine it; however, most virus preparations also contain a significant component of noninfectious material. Determination of the systemic distribution of this material would clearly be misleading when trying to maximize the plasma circulation of infectious virus. Issues such as these are a problem when using physical tracing methods such as quantitative (real-time) polymerase chain reaction (Q-PCR) or radiotracing techniques. Both techniques would produce misleading results when the virus preparation contains noninfectious virus particles, whereas Q-PCR would also be vulnerable to the presence of free DNA and radiotracing to the presence of free capsid proteins. These issues are likely to be significant using most conventional preparations of adenovirus.

The obvious alternative to following the distribution of virus DNA or proteins is to use virus infectivity as the marker for biodistribution, and in many situations, this can be useful. However, it fails when components of the experimental milieu interfere with infectivity, e.g., antiadenovirus-neutralizing antibodies, or where the virus of interest is tropism-ablated to prevent infection through the normal route. These different tracing techniques all find important

and complementary applications, but it is essential to define precisely how individual experiments were performed before the data generated can be interpreted accurately. We have tried to bear this caveat in mind throughout interpretation of data reported in this review.

Adenovirus pharmacokinetics in mice

Animal models have illustrated that hepatic uptake of systemically injected adenovirus is very efficient. It is widely accepted that adenovirus is rapidly taken up into Kupffer cells (KCs), significantly reducing the number of infectious virus present in the blood. One of the best studies in this field was performed by Alemany et al,[1] where the amount of infectious virus present was determined by isolating plasma and using it to infect 293 cells *in vitro*. These studies demonstrated that the half-life of adenovirus type 5 (Ad5) in mice was less than 2 minutes.[1] A recent report has investigated virus uptake into KCs using vectors labeled with the fluorophore, Cy3. These studies indicate that low doses of adenovirus (up to 3×10^{10} viral particles) are rapidly sequestered by KC, but higher virus doses can overcome this biological filter effect, leading to a nonlinear dose response in hepatic gene transduction.[2] The importance of the liver in determining the fate of intravenously administered adenovirus has been demonstrated by bypassing the hepatic circulation by clamping the portal vein, hepatic artery, and bile duct. While the clamp was in place, circulating plasma levels of infective virus remained constant but decreased rapidly following removal of the clamp.[3]

It should be noted that all of these pharmacokinetic studies were performed in normal mice, which would not have previously been exposed to human adenoviruses. It is likely that in animals with preexisting antiadenovirus antibodies, the infectious virus in plasma would be neutralized. A recent study has investigated the effects of preimmunization on virus biodistribution in a syngeneic mouse tumor model.[4] TM-40D mouse mammary tumors were grown in BALB/C mice. Animals were immunized with an intratumoral (i.t.) injection of 1×10^{11} viral particles of replication-defective Ad5 lacking a trangene (Ad-null). Two weeks later, the animals were given an i.t. injection of Ad luciferase, and virus biodistribution was examined and compared to the findings in naïve mice. As expected, preimmunization resulted in a significant decrease in luciferase gene expression in all tissues examined and also led to a fall in the duration of transgene expression. Unexpectedly, viral toxicity was noticeably greater in the preimmune animals: administration of 6×10^{11} virus particles i.t. caused death in 60% of the preimmunized group compared to just 15% of the naïve mice. The major toxicities were hepatitis and elevated liver enzymes. The increased acute toxicity observed in the preimmunized animals was unexpected as the humoral immunity was predicted to exert a protective effect against virus toxicity by inhibiting successful transduction of the liver and peripheral organs. The precise reason for greater toxicity in preimmunized animals is unclear at present, though if the effect is found to be reproducible, it may have significant implications for transfer of the approach into the clinic.

Virus retargeting strategies

Adenoviral vectors enter cells in a multistage process. First, the knob domain of fiber binds the Coxsackie and Adenovirus Receptor (CAR),[5] followed by an interaction between the RGD motif of the penton bases with $\alpha v\beta3$ and $\alpha v\beta5$ integrins, facilitating internalization of the virus by receptor-mediated endocytosis.[6] More recently, the involvement of a third cell surface interaction, with heparan sulfate glycosaminoglycans (HSGs), has been demonstrated.[7,8] CAR is widely expressed on many cell types, and it is thought that the rapid accumulation of systemically administered adenoviral vectors in the liver of mice is due to high hepatic levels of CAR and αv integrins.[9]

There is some disagreement regarding the effects of CAR binding on the biodistribution of systemically delivered adenoviral vectors in mice. Some studies have demonstrated that ablation of CAR binding does not alter the biodistribution or toxicity of systemically administered adenovirus in mice.[10,11] In both studies, the biodistribution was monitored by Q-PCR and transgene expression assays. A more recent report has demonstrated that a CAR-ablated vector with the KO1 mutation in the AB loop gave higher levels of hepatic gene transduction than vectors containing wild-type fiber.[12] These data suggest that CAR binding may not be the major determinant of hepatic transduction *in vivo* and, therefore, that other aspects of the viral entry mechanism such as integrin or HSG binding may play a significant role. The situation is not completely clear, however, because Einfeld et al[13] described a four-amino-acid substitution in the AB loop of adenovirus fiber that decreased liver transduction 10-fold.

To completely ablate the natural tropism of adenoviral vectors, both CAR and integrin binding sites must be mutated. Doubly ablated adenoviral vectors show up to a 700-fold reduction in luciferase gene expression in the liver following systemic administration.[13] Only Alemany and Curiel[10] examined the levels of CAR-ablated virus in the blood and showed, using a modified plasma infectivity assay, that the levels of CAR-ablated virus present in the blood were approximately 10-fold higher than unmodified virus over a 60-minute time period.

In order to target tumor cells specifically, CAR, integrin, and HSG binding must be abolished and replaced with a tumor-specific targeting mechanism. This can be achieved in several ways. A popular approach involves complexing the adenovirus with a bispecific molecule that blocks the interaction with CAR and redirects the virus to a novel receptor.[14] The CAR-blocking component of the bispecific molecule can be a neutralizing antibody, a soluble form of CAR, or a high-affinity peptide that binds to the knob. The receptor-binding component can be an antibody or fragment of an antibody, a high-affinity peptide, or even a small molecule that binds to the receptor. This approach has been used extensively to retarget replication-deficient adenoviral vectors. Ligands that have used in this approach include CD3,[15] E-selectin,[16] αv integrins,[16] FGF receptor,[17,18] and EGFR.[19]

An alternative approach involves genetic modification of the adenovirus vector to incorporate targeting sequences,

with the possibility of simultaneously ablating interactions of the fiber and penton base with their native receptors. High-affinity peptide ligands have been inserted into the HI loop or on to the C-terminus of fiber, into the RGD loop of penton base, or even into an exposed loop of hexon. Several peptide ligands have been identified, which are home to particular organs including vascular endothelium,[20] kidney,[21] head and neck,[22] and ovarian tumors.[23] The pharmacokinetics and biodistribution of the retargeted viruses do not appear to differ significantly from unmodified virus. The virus is cleared rapidly from the blood as determined by Q-PCR and gene expression data, and is found predominantly in the liver, with the exception of FGF2-retargeted adenovirus, which showed 10- to 20-fold less liver uptake than unmodified virus controls. This decreased liver uptake translated into a significant reduction in subsequent toxicity as measured by serum transaminases and histopathology in mice.[24]

Probably the most promising *in vivo* results to date have used adenovirus targeted with a bispecific antibody to angiotensin-converting enzyme (ACE).[25] The vector showed at least a 20-fold increase in viral DNA and transgene expression in the lungs compared to an untargeted virus following systemic administration in rats. Targeted gene delivery through ACE may offer new therapeutic opportunities in diseases such as pulmonary hypertension, pulmonary thromboembolic disease, and pulmonary carcinoma. However, although adenoviral vectors retargeted to infect through specific receptors give promising results *in vitro* and following local administration *in vivo*, the clearance times following intravenous administration are generally short. Bloodstream circulation must be significantly extended if the vectors are to provide any realistic platform for targeted delivery of genes to disseminated targets to mediate useful therapy.

Another approach to link targeting ligands to adenoviral vectors involves the use of polyethylene glycol (PEG). This strategy has important implications because PEGylation also provides a degree of protection from neutralizing antibodies. Heterobifunctional PEG has been used to modify adenovirus with a peptide designed to target ciliated epithelial cells[26] and a urokinase plasminogen activator–derived peptide.[27] In both of these cases, the tropism of the vector was expanded, but native tropism was not ablated. Alemany et al[1] studied the blood clearance rate of PEGylated adenoviral vectors in BALB/C mice. Infectivity of the PEGylated virus was measured by serial dilution of plasma and infection of A549 cells. A 300- to 1000-fold loss of infectivity was observed using modification under the conditions defined as "4% PEG" and the blood clearance rate of PEGylated virus during the first 30 minutes (determined using *ex vivo* infectivity and corrected using the 300–1000 factor noted above) was 4-fold slower than for normal virus. There was no evidence of toxicity with the PEGylated virus.

Fisher et al[28] showed that polymer modification of adenoviral vectors using multivalent polymers based on poly [*N*-2(hydroxypropyl)methacrylamide] (HPMA) was an effective means to ablate normal virus tropism, decreasing viral transgene expression by 100-fold. The polymer-coated virus could be retargeted to infect through novel receptors using FGF2 and vascular endothelial growth factor (VEGF). The vector exhibited ligand-mediated, CAR-independent gene transfer as well as resistance to neutralizing antibodies.[28] Polymer modification results in extended blood circulation times in mice and reduces hepatic gene expression by three logs.[29] Virus levels in the blood have been measured using ^{125}I-labelled virus, Q-PCR, and infectivity assays. While our findings are in accord with previous reports regarding the rapid clearance of infectious virus from the circulation, our Q-PCR and radioactivity results suggest a higher proportion of inactive virus is present in whole blood than in plasma. We believe this may involve interactions between the virus and blood components including erythrocytes, and leads to the observation that kinetic studies that work with only serum or plasma may be missing a significant proportion of blood-borne virus.

Conditionally replicating vectors

The use of replicating vectors, which may only infect a small proportion of tumor cells but are designed to allow spread of the virus to neighboring cells, can significantly increase the efficacy of gene delivery coupled with direct cytolytic activity. Replication competence can be made conditional on the biology of the cell infected; e.g., key virus proteins can be placed under the regulatory control of cell-specific gene promoters, restricting virus production to the appropriate target cells. This approach is considered in more detail later. A more sophisticated system, however, has been to modify the adenovirus proteins to enable the virus to replicate only in cells that bear cancer-related phenotypes. Two main approaches have been pursued, and these are considered briefly here.

Adenoviruses deficient in E1B

Inhibition of p53 protein activity must be blocked in order to allow efficient viral replication. The 55-kDa protein encoded by the *E1B* region of normal adenovirus binds and inactivates p53, allowing replication in normal cells. ONYX-015 *(dl1520)* contains a 827-bp deletion in the *E1B* region of the viral genome and a point mutation, generating a premature stop codon that prevents expression of a truncated form of the E1B–55-kDa protein.[30] It follows that infection of normal cells with ONYX-015 should generate a p53 response that leads to growth arrest or apoptosis, preventing virus replication. In contrast, tumor cells lacking a functional *p53* gene should be unable to suppress viral replication. In this way, replication of ONYX-015 should be restricted to p53-deficient cells, resulting in selective destruction of cancer cells.[31]

However, several recent reports indicate that ONYX-015 can replicate in a variety of tumor cell lines with wild-type p53.[32–34] p14ARF[35] is a tumor suppressor protein that binds to Mdm2, sequestering it in the nucleolus and preventing degradation of p53.[36] A recent study has demonstrated that lack of p14ARF expression in tumors with wild-type p53 disrupts the p53 signaling pathway, leading to high and uncontrolled Mdm2 protein activities facilitating

ONYX-015 replication. Deregulation of Mdm2 prevents p53 from exerting its protective effects after adenoviral infection. Reintroduction of functional p14ARF into tumor cells caused induction of p53 and prevented replication of ONYX-015, but not wild-type adenovirus. These findings indicate that other defects within the p53 pathway, other than mutations of p53 itself, can render cells permissive for ONYX-015.[37]

Adenoviruses deficient in E1A

Other replication-competent viruses have been generated by mutating the *E1A* region of the adenoviral genome. KD1 and KD3 are vectors containing deletions in the *E1A* gene, which abolish the ability of *E1A* to deregulate the cell cycle and cause cells to move from G_0 to S phase. The E3 region genes that protect virus-infected cells from destruction by the immune system have also been deleted to prevent runaway viremia by the vector. These viruses can also overexpress the adenovirus death protein (ADP), which gives them increased capacity for cell lysis and virus spread.[38] The vector can also be modified to include the tissue-specific surfactant protein B (SPB) promoter, which is expressed in type II alveolar and bronchiolar epithelial cells.[39] KD1-SPB replicates well in H441 papillary lung adenocarcinoma cells but very poorly in Hep3B hepatocellular carcinoma cells, which do not express SPB, and the virus has been shown to suppress growth of H441 tumors in nude mice.[40] Recently, it has been demonstrated that coadministration of KD1 or KD3 with replication-defective adenoviral vectors increases cytotoxicity and transgene expression.[41] Systemic administration of the KD vectors has not yet been reported; whereas the kinetics of the input virus would be expected to be the same as a replication-incompetent virus, any small amount of virus gaining access to permissive cells might modify the pattern of *in vivo* transduction considerably.

Several *E1A* mutant adenoviruses have been described: *dl312* is totally *E1*-deleted, *dl101* has a deletion in the p300-binding region,[42] whereas *E1A* CR-2 contains deletions in the conserved region of *E1A*.[43] However, a virus with a mutation in the pRB family-binding region of *E1A* (*dl922–947*) has demonstrated the best antitumor efficacy in animal models. MDA-MB-231 human breast cancer cells were injected orthotopically into nude mice, in a model system that forms metastases in the lungs and lymph nodes as well as the primary breast tumor. Mice were treated with *dl922–947*, *dl1520* (ONYX-015), or wild-type Ad5 by tail vein injection (2×10^8 pfu daily for 5 days). They were sacrificed when the primary tumor reached 1200 mm^3 or at 3 months, and examined for the presence of metastases. Animals treated with *dl922–947* had significantly fewer metastases (1/19) compared with *dl1520* (12/19) or wt virus (6/18).[44] However, no viral pharmacokinetics or biodistribution studies were performed.

Tissue-selective promoters for creation of conditionally replicating adenovirus

Replication-competent viruses can be targeted by the use of tissue-specific promoters to restrict adenoviral replication to specific tissues. Prostate-specific antigen (PSA) is a widely used marker for the diagnosis and management of prostate cancer. Enhancer/promoter constructs derived from the 5′regions flanking the human *PSA* gene have been cloned upstream of the *E1A* gene to generate CN706, a prostate-specific enhancer containing provisionally replicating adenovirus. A single i.t. injection of virus led to destruction of LNCaP xenografts and abolished PSA production in a nude mouse model.[45] A phase I trial of CN706 has recently been completed in patients with locally recurrent adenocarcinoma of the prostate. Up to 10^{13} viral particles were administered into the prostate using brachytherapy techniques. Treatment was well tolerated and some patients achieved biochemical (PSA) responses.[46] Provisionally replicating *E1A* mutant viruses containing the AFP promoter[47] and the DF3/MUC1 promoter[48] have been shown to have antitumor activity in animal models of hepatocellular carcinoma and breast cancer. In these studies, the virus was administered by i.t. injection and virus biodistribution and pharmacokinetics were not addressed.

Immune responses

Another factor that needs to be addressed before adenoviral vectors can routinely be used systemically is provocation of the immune system. Most patients have previously been exposed to adenovirus and have pre-existing neutralizing antibodies. In the clinical trials using ONYX-015, the majority of patients presented with neutralizing antibodies and almost all showed a significant increase in titer after the initial virus injection.[49,50] There are also significant concerns over vector immunogenicity following the death of a patient after hepatic artery infusion of a replication-defective Ad5 vector.[51,52] It is thought that viral capsid proteins are involved in the acute cytokine release observed shortly after virus administration.[53] Inflammation then occurs at the site of gene transfer due to T- or B-cell–mediated targeting of transduced cells.[54,55] A recent study has demonstrated that both neutralizing and non-neutralizing antiadenovirus antibodies are capable of activating complement.[56] Plasma from healthy volunteers was incubated with increasing doses of adenovirus and the levels of C3a measured. There was a dose-dependent activation of C3a but levels varied significantly between subjects. The report indicates that performing *in vitro* complement studies on patients undergoing gene therapy may be useful and suggests the use of prophylactic steroids (to reduce subsequent inflammation) or C1 esterase inhibitors.

Systemic delivery of adenovirus in clinical trials

The only clinical trials to date involving systemic delivery of conditionally replicating viruses are the ONYX-015 studies. The virus has been administered by hepatic artery infusion for the treatment of metastatic colorectal cancer[57] and intravenously in patients with metastatic lung tumors.[58] Both studies were dose escalation regimens. No dose-limiting toxicity was identified, and the toxicity profile was not altered when the virus was coadministered with

chemotherapy. The most common side effects were mild to moderate fever, rigors, and a dose-dependent transient transaminitis. Neutralizing antibody titers were significantly increased. Evidence of viral replication in the blood was detectable in patients receiving virus doses $>2\times10^{11}$ particles and i.t. replication was confirmed in one patient in the intravenous delivery trial. No objective tumor responses were demonstrated with ONYX-015 alone, but a partial response and tumor stabilization were observed in patients who had been treated with high doses of ONYX-015 and chemotherapy. These studies have demonstrated that intravascular infusion of ONYX-015 was well tolerated and resulted in viral infection of metastatic pulmonary tumors and i.t. viral replication, suggesting that systemic delivery of genetically modified adenoviral vectors is a feasible approach.

Pharmacokinetic studies, using Q-PCR, were performed to detect viral genomes in plasma. In the intrahepatic artery trial, no virus could be detected after 6 hours;[57] but in the intravenous administration protocol, low levels of virus ($\sim2\times10^6$ virus particles/mL, approximately 0.3% of the total injected dose) were detectable up to 6 hours after the end of virus infusion in patients receiving high doses of virus (2×10^{12} particles). These studies were performed on patients that were initially antibody-negative. However, repeated administration did not seem to affect the pharmacokinetic data; virus could still be detected in the blood 6 hours after the fourth virus infusion. The titers of viral DNA were independent of dose over the range of 2×10^{10}–6×10^{11} particles. At 2×10^{12} particles and above, there appeared to be a greater plasma PCR signal, albeit not statistically significant. The half-life of the viral DNA was about 20 minutes over the first 2 hours following injection, although infectivity was not assessed, and levels did not change as a function of time from 2 to 6 hours after treatment.[58]

Key hurdles to overcome

There are several approaches that could be used to increase the clinical efficacy of the conditionally replicating viruses. Preclinical studies have demonstrated that up to 80% of ONYX-015 is taken up into the livers of mice following intravenous injection.[49] It is likely that this figure will be even greater in humans; hence, hepatic uptake needs to be prevented in order to permit effective targeting to metastatic tumors. A previous study in mice has shown that depletion of KC using gadolinium chloride resulted in an increase in plasma virus levels and a reduction in hepatic gene expression.[1] However, it is unlikely that this approach would be used clinically.

Any virus that is delivered systemically must be protected from neutralizing antibodies present in the sera of most patients. Genetic strategies are generally inefficient at overcoming this problem, and surface modification of adenovirus with reactive hydrophilic polymers is presently the most effective way of preventing unwanted neutralization. The use of HPMA to coat replication-deficient adenovirus can reduce CAR-mediated entry into cells, ablates unwanted hepatic gene expression in mice, allows versatile retargeting, and simultaneously protects from neutralizing antibodies.[28,29] This technique could easily be extended for use with replication-competent viruses.

New versions of the ONYX-015 that contain prodrug-converting enzymes in order to generate high local concentrations of cytotoxic compounds following systemic administration of nontoxic prodrugs have been developed. An E1B–55 kDa–deleted adenovirus has been devised, expressing the herpes simplex virus thymidine kinase (Ad.TKRC).[59] Ad.TKRC was evaluated in nude mice bearing subcutaneous xenografts of human A375 melanoma and ME180 cervical carcinomas. The i.t. injection of Ad.TKRC followed by treatment with ganciclovir (GCV) resulted in a significant increase in the survival times of tumor-bearing mice. Treatment of tumors with Ad.TKRC without GCV resulted in a similar antitumor effect, confirming that the replicating vector has an oncolytic effect. These results confirm that both the oncolysis caused by a replicating virus and suicide/prodrug gene therapy with HSVtk/GCV have potent antitumor effects. When combined, these two approaches are complementary, resulting in a significantly improved treatment outcome.

Conclusions

Improved systemic delivery of adenovirus will require novel coat modifications to prevent unwanted infection of nontarget cells, as well as evasion of neutralization by anti-adenovirus antibodies. The possibility of virus interactions with blood cells should also be considered, with kinetic studies being performed in both plasma and whole blood. Identification of the viral genes and immune parameters effecting efficacy and toxicity may lead to the development of new immunomodulatory strategies, whereas clearer understanding of the synergy between replicating adenoviral vectors and chemotherapy should enable production of systemically deliverable vectors with true clinical efficacy.

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Replicative adenoviruses for cancer therapy

Ramon Alemany, Cristina Balagué, and David T. Curiel

*Division of Human Gene Therapy, Departments of Medicine, Pathology, and Surgery, Gene Therapy Center, University of Alabama at Birmingham, Birmingham, AL 35294-3300. *Corresponding author (david.curiel@ccc.uab.edu).*

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Rapid advances are being made in the engineering of replication-competent viruses to treat cancer. Adenovirus is a mildly pathogenic human virus that propagates prolifically in epithelial cells, the origin of most human cancers. While virologists have revealed many details about its molecular interactions with the cell, applied scientists have developed powerful technologies to genetically modify or regulate every viral protein. In tandem, the limited success of nonreplicative adenoviral vectors in cancer gene therapy has brought the old concept of adenovirus oncolysis back into the spotlight. Major efforts have been directed toward achieving selective replication by the deletion of viral functions dispensable in tumor cells or by the regulation of viral genes with tumor-specific promoters. However, the predicted replication selectivity has not been realized because of incomplete knowledge of the complex virus–cell interactions and the leakiness of cellular promoters in the viral genome. Capsid modifications are being developed to achieve tumor targeting and enhance infectivity. Cellular and viral functions that confer greater oncolytic potency are also being elucidated. Ultimately, the interplay of the virus with the immune system will likely dictate the success of this approach as a cancer therapy.

Keywords: replicative adenovirus, virotherapy, oncolysis

The concept of virotherapy, an approach to the treatment of cancer with viruses, was inspired early in this century by the observation of occasional tumor regressions in cancer patients suffering from virus infections or receiving vaccinations[1]. Many different viruses were subsequently tested in clinical trials as lytic agents of tumor cells, but a low efficacy/toxicity ratio led to their abandonment. Soon after its isolation in 1953, adenovirus was also tested as an oncolytic agent because of its prolific growth in human epithelial cell lines. In this early trial, cervical carcinomas of 30 patients were injected with different adenovirus serotypes. The results paralleled those obtained with other viruses: an initial tumor regression followed by tumor progression, with a response inversely correlated with the antiviral immune response[2]. The lack of evident therapeutic efficacy also led investigators to dismiss adenovirus as an antitumoral agent.

Despite this initial disappointment, several viruses have recently come forth again as promising anticancer agents. The increasing knowledge about virus–cell interactions has shed light on the natural tropism of some viruses toward tumor cells. For instance, reovirus requires an activated *ras* pathway for infection[3], whereas the autonomous parvovirus life cycle is limited to actively replicating cells[4]. Likewise, several natural and engineered mutants of the herpes simplex virus type 1 can replicate only in dividing cells[5]. Adenovirus has also emerged as a virus that can be engineered with oncotropic properties, as a result of increasing knowledge of adenoviral interactions with cell cycle regulatory proteins and the experience gained from its use as a gene delivery vehicle. Even though the adenovirus tumor selectivity can be tackled at different levels, perhaps the area that has concentrated most research efforts so far is the development of conditionally replicative adenoviruses (CRAds) designed to replicate exclusively in tumor cells. Improving the delivery of CRAds, both to local-regional and disseminated disease, as well as the virus intratumoral spread are growing research areas. Last, but not least, the study of the interaction of replicative adenoviruses with the immune system is mandatory in order to improve the outcome of viral oncolysis. Here, we review the studies performed on replicative adenoviruses at these different levels and provide some insights for future studies (see Fig. 1).

Adenovirus tumor-selective replication

Since the leading efforts of Onyx Pharmaceuticals (Richmond, CA), the study and design of adenoviruses that replicate selectively in tumor cells is the area of most intensive research in adenovirus-based cancer virotherapy[6]. Before analyzing what has been achieved in selective replication, it is useful to comment on the assays that have been employed.

The selective replication of a CRAd has been generally studied by comparing different human cell lines in vitro. Whereas different cell lines may show various levels of susceptibility to adenovirus infection and virus production, these variables have not always been considered. This deficiency can be corrected by including a comparison with a nonselective adenovirus like the wild type (Adwt) (see below), albeit not all studies have incorporated this control. The effect of a CRAd in vitro has been measured at the level of viral DNA replication, late gene expression, cell death, and progeny production. These parameters are not always correlated. Viral DNA replication, hexon expression, and cell death can occur without progeny production. Conversely, in some cell types, such as fibroblasts and keratinocytes, virus production can occur without evident cytopathic effect. Therefore, although crystal violet staining, trypan blue exclusion, and other live-dead assays yield information about the CRAd effects in each cell type, from an oncolysis standpoint progeny production is most relevant. Furthermore, the progeny released to the supernatant is likely to have more oncolytic predictive value than if we measure it after releasing the virus from the cells by freeze/thaw cycles. For a given cell type, the burst size or number of infectious particles produced per cell is a quantitative measure of progeny production.

Perhaps the best and simplest way to numerically express the selectivity of a CRAd between a tumoral and a normal cell line, could be the following:

$$\left(\frac{\text{CRAd burst size in tumor cell}}{\text{CRAd burst size in normal cell}}\right) \times \left(\frac{\text{Adwt burst size in normal cell}}{\text{Adwt burst size in tumor cell}}\right)$$

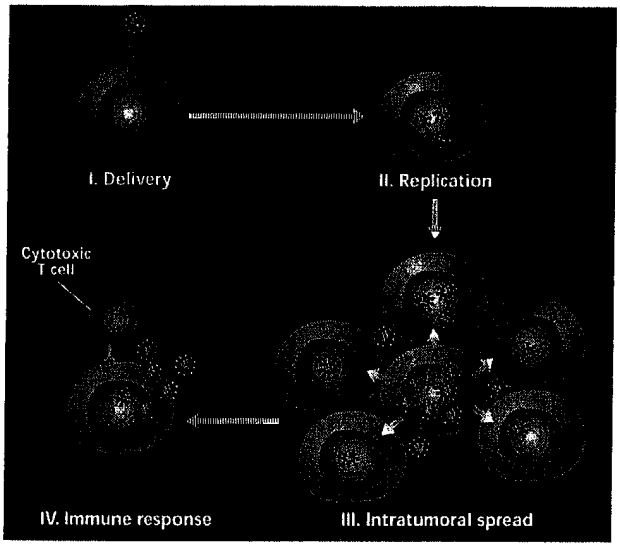

Figure 1. Oncolysis steps that provide opportunities for intervention.

Using this formula, if a CRAd produces 1,000 plaque-forming units (p.f.u.)/cell in a tumor cell and 1 p.f.u./cell in a normal cell, and Adwt produces 10,000 and 100, respectively, then this CRAd is 10-fold more selective than Adwt. The presence of Adwt in this formula corrects for difference in infectivity and virus production between the different cell types. On the other hand, the choice of normal cells is critical. Primary human embryo kidney cells are the most sensitive to adenovirus types 2 and 5 infections[7]. Human fibroblast cell lines and endothelial cell lines, employed in different studies[8–10], show poor infectivity and delayed cytopathic effect. Mammary (MEC) and bronchial (NHBE) epithelial cells used in other reports[9,11] are more permissive to adenovirus infection and production. In fact, the effect of the CRAd on normal cells, particularly those of epithelial origin that could become infected when targeting a particular tumor, should be analyzed although their availability has so far limited such studies.

Many levels in adenovirus replication may be regulated for the purpose of generating a CRAd. In recent years, two major strategies have come forth. In the first one, viral genes that become dispensable in tumor cells, such as the genes responsible for activating the cell cycle through p53 or Rb binding, have been completely or partially deleted. In the second strategy, transcription of viral genes has been controlled by replacing the native viral promoters with tumor-specific promoters (tsp) (see Table 1 for the different types of CRAds developed so far). Mutants defective at other levels such as intracellular trafficking, nuclear import of the viral genome, RNA splicing, nuclear export of RNA, or protein translation are conceptually CRAd candidates. For example, a virus in which the splicing of a viral gene or an interfering stop signal is regulated like the tumor-associated splice variant of CD44 could be tumor selective[12]. Nonetheless, we have learnt that, in reality, achieving tumor-selective replication is not so simple. McCormick's group at Onyx proposed that an E1b-55kDa-deleted adenovirus would replicate selectively in *p53*-deficient cells, an alteration common in tumors. The protein encoded by the *E1B 55K* gene binds and inactivates *p53* in normal cells in order to initiate virus replication. Therefore, only cells that have lost *p53* are permissive for CRAd replication, because there is no requirement for the viral E1b-55kDa protein in switching off *p53*[13]. When a few cell lines were compared, this was found to be the case.

In *p53*$^-$ cell lines C33A and U373, the mutated virus dl1520 (Onyx-015) was shown to lyse cells at similar levels as the wild-type virus Ad5wt, but was 100-fold less effective in lysing the *p53*$^+$ cell line U87. The same result was observed for the matched pairs of cell lines U2OS55K/U2OS and RKOp53/RKO[13]. However, lytic assays from the same group revealed exceptions to the lack of replication of dl1520 in *p53*$^+$ cell lines, such as HepG2, HlaC, and HCT116 (ref. 9). When titering the viral progeny, Rothmann and colleagues[11] have also reported a lack of correlation between p53 status and dl1520 replication[11]. Contrary to the conclusions from lytic assays, U373 (*p53*$^-$) produced 100-fold lower levels of dl1520 than wild-type adenovirus, and U87 (*p53*$^+$) produced as much dl1520 as the wild type. In this report, the amount of transcriptionally active p53 was determined using a p53-responsive reporter plasmid, to rule out the possibility that *p53*$^+$ cell lines could have p53 inactivated through mechanisms such as *MDM2* overexpression. Furthermore, in *p53*$^-$ cells as well as normal primary cells, the differences in progeny production were reduced when infecting with more infectious particles per cell, confirming the fact that E1b becomes dispensable at high multiplicities of infection.

Hay and colleagues[8] have studied viral DNA replication, viral protein synthesis, host cell protein shutoff, cytopathic effect, and progeny production of the dl338 E1b-55kDa mutant, and found no correlation with p53 status. Besides a lack of correlation between dl1520 progeny production and p53 status, Goodrum and Ornelles[14] have also described that S-phase cells are more susceptible than G1-phase cells to cell killing by dl1520, but not wild-type adenovirus[14]. These observations seem to be related to other functions of the E1b-55KDa protein. The initial studies of this viral protein indicated that *E1B 55K* mutants are impaired in p53-defective cells[15,16]. The defect is attributed to the reduced rate of late viral protein synthesis due to impaired nuclear RNA export mediated by the E1b-55kDa–E4-34kDa complex. A defect in viral mRNA translation can also account for the p53-independent impairment of dl1520[17].

It is conceivable that cells in S phase are less dependent on this complex to export or translate viral RNA, and thus dl1520 has certain specificity for dividing cells. Mutants affected in *p53* binding, but not in other E1b-55KD functions, may result in the desired conditional progeny production upon p53 absence. However, these functions are not easily separable in the protein sequence. Another step toward restricting replication to p53-deficient cells may be the deletion of the E4-34kDa protein domains that also inactivate p53. On the other hand, the role of p53 in adenovirus propagation is a question that remains to be answered. If p53 is necessary for efficient release of progeny from the infected cell[18], other genes involved in cell lysis that can complement the p53 defect will need to be incorporated into CRAds designed for *p53*$^-$ tumors.

Currently, dl1520 (ONYX-015) has reached phase I and II clinical trials for head and neck, pancreatic, ovarian, colorectal, lung, and oral carcinomas. In these trials, up to 2×10^{13} viral particles have been administered systemically or locally, but have brought about no objective tumor responses[6]. Efficacy rather than toxicity seems to be the limitation. Combination with chemotherapy or the insertion of therapeutic genes in the virus have increased the efficacy in animal models[19,20]. Incorporation of the thymidine kinase gene in a CRAd has been shown to confer therapeutic efficacy and safety traits. However, the effects of radiation, chemotherapy, or cytotoxic gene therapy on viral replication and the timing of these auxiliary interventions with respect to the oncolytic treatment have not been evaluated yet, and it would be also desirable to understand the limitations of the single agent before trying complex combinations.

A mutant also proposed for specific replication is based on the deletion of the retinoblastoma gene (*Rb*)-binding site of E1a[21]. These mutants cannot induce resting cells to pass the G2/M checkpoint and progress to mitosis[22]. One of these mutants AdΔ24 has been studied for oncolysis of glioblastomas. Cells arrested by the previous infection with a pRb-expressing adenoviral vector become refractory to cell lysis by AdΔ24. In a brain tumor context where normal cells are resting, the specificity of this agent could be enough to allow a certain level of amplification in the dividing tumor cells. Mutants unable to bind Rb and p300 will likely be even more tumor-selective because of their inability to induce S phase. The lack of good animal

Table 1. Types of adenoviruses used as oncolytic agents

Name (serotype)	Basis of tumor-selective propagation	Therapeutic traits	Reference
Ad wild type (various serotypes)	None	Oncolysis	2, 51
Ad5/IFN (Ad5)	None	Oncolysis & immunostimulatory gene therapy	54
dl1520 or Onyx015 (Ad2/5)	E1b55kDa-deletion abrogates *p53* binding	Oncolysis	13
AdTK^{RC}	E1b55kDa-deletion abrogates *p53* binding	Oncolysis & suicide gene therapy (TK)	19
Ad-5-CD-TKrep or FGR (Ad5)	E1b55kDa-deletion abrogates *p53* binding	Oncolysis & suicide gene therapy (CD + TK)	55
AdvE1AdB-F/K20 (Ad5)	E1b55kDa-deletion abrogates *p53* binding	Oncolysis with enhanced infectivity	41
AxE1AdB (Ad5) & AdCAhIL-2 (Ad5)	E1b55kDa-deletion abrogates *p53* binding	Oncolysis & immunostimulatory gene therapy	31
AdD24 (Ad5)	E1a deletion abrogates *Rb* binding	Oncolysis	21
CN706 (Ad5)	Regulation of E1a under the PSA promoter	Oncolysis	24
CN763 (Ad5)	Regulation of E1a under the kallikrein 2 promoter	Oncolysis	10
CN764 (Ad5)	Regulation of E1a under the PSA promoter and E1b under the kallikrein 2 promoter	Oncolysis	10
CV739	Regulation of E1a under rat probasin promoter and E1b under human PSA promoter	Oncolysis	29
CV787	Regulation of E1a under rat probasin promoter and E1b under human PSA promoter	Oncolysis (enhanced compared with CV739 due to the presence of E3)	29
AvE1a041	Regulation of E1a under the AFP promoter	Oncolysis	23
GT5610 (Ad5) + AdHB (Ad5)	Regulation of E1a under the AFP promoter	Oncolysis	30
Dl337 (Ad5)	None	Oncolysis (enhanced due to E1b-19kDa deletion)	43
Dl316 (Ad5)	The complete deletion of E1a makes this mutant dependent on intrinsic or IL-6-induced E1a-like activity	Oncolysis	56
Dl118 (Ad5)	The complete deletion of E1b abrogates p53 binding; however, E1a-induced apoptosis is not inhibited by E1b-19kDa	Oncolysis	44

models that allow adenovirus replication in normal cells is an obstacle to evaluate the toxicity of this and other CRAds.

A second strategy to achieve tumor-selective replication is the replacement of viral promoters with tumor or tissue-specific promoters. Paul Hallenbeck (Genetic Therapy–Novartis; Gaithersburg, MD) and Daniel R. Henderson (Calydon; Sunnyvale, CA) have pioneered the efforts in this direction using α-fetoprotein (AFP) and prostate-specific antigen (PSA) promoters to drive the adenovirus *E1a* gene[10,23,24], to treat hepatocellular and prostate carcinomas, respectively. Even though they have shown some degree of specificity with these promoters, it is known that cellular promoters do not keep the proper fidelity in the viral genome[25]. Low levels of viral products such as E1a may be sufficient for replication, thus preventing specificity. Different mechanisms can account for this leakiness: the presence of enhancers in the viral genome, the presence of viral DNA-binding protein instead of histones, and the location of the viral genome in active centers of transcription, among others. Insulation of promoters with genomic DNA sequences has been shown to increase the promoter fidelity[26].

In a different strategy, the interference of *cis* sequences has been avoided by delivering the regulated viral gene expression cassette in a plasmid, allowing only one round of viral replication[27]. The regulation of viral genes for which the products are needed in greater amounts, or of several viral genes that participate in one viral function (e.g., the role of E1b-55kDa and E4-34kDa in RNA transport), could further limit viral replication. In this regard, a double-regulated E1a/E1b adenovirus by two different prostate-specific promoters has been reported and shown greater attenuation in non-prostate tumor cells[10]. If promoter fidelity in the viral context can be improved, promoter regulation is attractive because it does not rely so much on our knowledge of viral functions. In addition, this strategy has the advantage, compared with E1a mutants, that the absence of E1a expression in normal cells may decrease E1a-associated toxicity.

In the strategies mentioned above, the size of the exogenous DNA that a CRAd can accommodate is limited. Whereas early region 3 is dispensable for virus replication, it is actually necessary for proper cell lysis[28] and to partially evade the immune system[29]. Therefore, a CRAd with no deletions can only accommodate an extra 1.8 kb of DNA. To avoid this limitation, a double system composed of both a gutless 36-kb capacity adenovirus containing an insulated AFP-E1 cassette and an E1-deleted adenovirus has been used and shown to selectively lyse AFP-expressing tumors[30]. In a similar complementation strategy, a CRAd such as Onyx-015 has been coinjected with an E1-deleted vector expressing interleukin-2 to increase intratumoral expression and therapeutic outcome[31]. However, the requirement of double infection curtails the efficacy at low multiplicities of infection as would be the case after systemic delivery, or in poorly infectable tumors.

Delivery and intratumoral spread

Although nonreplicative adenoviral vectors have been the subject of numerous modifications for efficient and selective delivery to tumors, little has been done on the delivery of replicative adenovirus. Nonetheless, the rules that apply to the delivery of adenoviral vectors can also be applied to CRAd delivery. A proper delivery should spare normal cells from infection to avoid toxicity, so it includes the concept of tumor targeting. The delivery requirements to local or disseminated cancer are, however, very different. For localized malignant disease, tumor targeting of replicative adenoviruses may not be critical. Local-regional ovarian carcinomas, head and neck, and brain tumors can be approached from a direct injection route with chances of success. For disseminated cancer, however, systemic delivery is necessary.

Adenovirus is not a blood-borne virus and its clearance from blood is very effective[32]. Like many other viruses, particles, and colloids, adenovirus is rapidly cleared by liver Kupffer cells by an unknown mechanism that seems independent of the interaction of its capsid fiber with the Coxsackie-adenovirus receptor (CAR). Other CAR-dependent cellular interactions, such as those with hepatocytes, could be bypassed by using CAR-binding ablated adenoviruses[33]. In this way, CAR-binding ablation could lessen the hepatocellular toxicity associated with adenovirus systemic delivery. Furthermore, other capsid modifications based on the alteration of hydrophobicity or charge are conceivable in order to increase virus persistence in blood, which in turn would facilitate virus delivery to tumors.

The porosity of the endothelial barrier could account for certain levels of passive tumor targeting, since, with the exception of spleen

and liver vessels, only tumor vessels may allow the extravasation of particles in the adenovirus size range. This is the principle behind the systemic delivery of drugs by long-circulating stealth liposomes[34]. On the other hand, active tumor targeting of adenovirus has been achieved using antibodies or other ligands, such as epidermal growth factor and basic fibroblast growth factor, attached to its capsid[35,36]. In vivo stability and size of these complexes are caveats that need to be addressed. A better strategy is to genetically modify the fiber or another exposed capsid protein to present a tumor-specific ligand. Progress in this direction has been encouraged by the successful insertion of small peptides such as the Arg-Gly-Asp (RGD) motif in the adenovirus fiber[37], resulting in adenovirus retargeting.

Finally, nonviral delivery of the replicative agent genome can be considered as another way to circumvent the blood clearance, targeting, and toxicity limitations associated with adenovirus particles. In this regard, linear adenovirus DNA is poorly infective, especially when the terminal protein attached to the ends that act as a replication primer is not preserved after DNA purification. Circular adenovirus genomes fused at their termini form infectious plasmids. If the infectivity of these plasmids could be increased by modifying the terminal repeats so they can efficiently dock the replication initiation complex, then these plasmids could be delivered by means of nonviral vectors optimized for systemic administration and tumor targeting.

Intratumoral spread is a problem different from delivery. As mentioned above, selective tumor delivery requires the ablation of the natural tropism of adenovirus, determined by its binding to CAR[33]. In contrast, to enhance intratumoral spread, it is desirable to broaden the tropism to different entry pathways, avoiding the selection of noninfectable tumor cells. Another difference is that, whereas delivery can be accomplished by conjugation of the virus to ligands, intratumoral spread will require the capsid modifications to be genetically incorporated in order to be present in the progeny. The function of the natural adenovirus type 5 receptor is not known, but its expression seems ubiquitous. However, some reports have indicated that in certain cases tumors may have low CAR levels[38,39]. To broaden the viral tropism, ligands have been incorporated into the fiber without interfering with CAR binding. The HI loop and the C terminus of the adenoviral fiber allow these insertions. The insertion of small peptides, such as stretches of lysines to bind heparan sulfate and polyanionic cellular receptors, as well as peptides containing the RGD motif to bind αv integrins have been shown to broaden viral tropism and increase infectivity[37,40]. In a CRAd context, the insertion of a polylysine at the fiber C terminus increased the oncolytic potency of Onyx-015 in vitro and in vivo by intratumoral administration in the glioblastoma cell line U373MG[41]. However, as described for several viruses, strains or mutants selected for enhanced or widespread binding in vitro may not target specific cell types after systemic administration[42].

Another approach to increase the viral spread has been to enhance the release of the replicative vector from the tumor cells. A replicative adenovirus deleted in the anti-apoptotic E1b-19kDa viral gene induces more apoptosis, is released earlier, and spreads faster than wild type[43]. The combined deletions of E1b-55kDa and E1b-19kDa could therefore enhance the oncolytic effect of E1b-55kDa mutants, such as Onyx015. An adenovirus containing this complete E1b deletion has been shown to be cytotoxic[44]. The effect of the E1b-19kDa deletion on progeny production is, however, variable among different cell lines, and the effect of premature apoptosis on progeny production remains an issue. Moreover, the possible induction of apoptosis in normal cells with the subsequent associated toxicity should be taken into account. Rather than deleting viral inhibitors of cell death, such as E1b-19kDa, one could seek to enhance the mechanism by which adenovirus promotes cell death. Preserving the adenovirus E3-11.6kDa death protein[28] in a CRAd has been shown to enhance its particle release and oncolytic potency[29]. Proteolysis of keratins by L3-23kDa and inhibition of cell translation by L4-100kDa also promote cell lysis and progeny release[45]. The dependence of the oncolytic potency of CRAds on these viral functions remains to be studied.

A different area of intervention to be explored is the diffusion of CRAds throughout the tumor. Several vasoactive drugs, cytokines, or physical treatments, such as radiation or heat, increase tumor vascular permeability and blood flow, leading to faster diffusion[46]. The incorporation of lytic enzymes, such as hyaluronidase, into a CRAd could also increase diffusion rates.

Control of the immune response

As mentioned earlier, the major obstacle for a successful virotherapy is the neutralizing immune response. In the early clinical trials with wild-type adenovirus, less frequent therapeutic responses were observed in patients with elevated serum neutralizing antibodies[2]. Cortisone was administered to lessen the immune reaction and although it seemed to enhance the initial intratumoral necrosis, it did not affect the antibody production. In Onyx-015 trials, where a well-purified virus has been used, it seems that at least pre-existing antibody levels have not influenced antitumoral activity or toxicity[6]. Although oncolytic/immune response kinetics need to be addressed meticulously in clinical trials to understand CRAd limitations, an area that requires improvement is the development of an appropriate preclinical model.

Preclinical studies on the interaction of the immune system with replicative adenoviruses have been limited by the lack of appropriate animal models, because of the poor replication of human adenovirus in other species, including monkeys. For this reason, only immunodeficient murine models have been employed to study oncolytic potency in xenografted human tumors. Perhaps the only preclinical studies on the effect of the immune system in virotherapy come from models of oncolysis by herpesvirus hrR3 in rodents, where the virus can replicate. In one report, innate preimmune IgM and neutralizing antibodies have precluded successful oncolysis of brain tumors in rats[47]. In contrast, no differences have been found treating liver metastases with hrR3 in immunocompetent or immunodeficient mice[5].

Ad5 has been administered oronasally into different species to reproduce the pathology of human infections. Cotton rats and pigs have been identified as the most permissive models for Ad5 replication, which occurs to some extent in lungs[48,49]. Although some conclusions could be obtained regarding the efficacy of oncolytic adenoviruses in these immune-competent hosts, these models are not permissive enough to test the toxicity of adenovirus, as indicated by the positive correlation between titers in tissues and input dose of Ad5. In truly permissive species, viral titers in tissues are independent of the dose injected. In general, murine and simian cells allow Ad5 DNA replication, but a late block prevents progeny production[7]. However, there is some evidence that mice support a low level of replication of Ad5 (ref. 50). After a high intravenous dose in mice and hamsters, Ad5 undergoes an abortive, but lytic, infection in many hepatocytes, whereas only a few are able to complete virus production at low levels[32]. Khoobyarian and colleagues[51] observed Ad2 progeny production in hamster melanoma cells and applied this model to oncolysis. Only high doses of Ad2 injected soon after the implantation of the tumors were effective. Poor permissiveness of hamster cells could account for this lack of efficacy.

In an attempt to find a good murine model for CRAds, Ganly and coworkers[52] recently have studied the replication of Ad2 in murine cells. Two cell lines, B9 and SN161, were shown to produce progeny at levels 50-fold and 25-fold (respectively) lower than the human A2780Cp cell line, but no comparison to the commonly used human 293 cell line was provided. This work may lead to immune-competent animal models where some virotherapy could be achieved, opening the possibility to study and modulate the immune system. It is conceivable that neutralizing antibodies will counteract the spread of the virus and antiviral cytotoxic T lymphocytes will destroy infected cells. In this case, shifting the immune response toward the

T-helper 1 type could change the oncolytic outcome. Interleukin-12 can induce this shift and has been shown to abrogate the development of neutralizing antibodies against adenoviral vectors[53].

Conclusions

Replicative adenoviruses are being engineered to achieve selective targeting and amplification for the treatment of local and disseminated cancer. Whereas chemotherapy and immunotherapy remain the current therapeutic choices for disseminated tumors, an agent that can be delivered systemically, can be targeted to tumor cells, and can amplify their cytolytic effect in a tumor-specific manner would undoubtedly be of clinical benefit. These agents share some attributes of the immune system with the advantage that tumors have not been exposed to them before their administration and thus have not selected specific mechanisms to evade them. Blood persistence, tumor targeting, tumor-specific replication, lateral spread, and the interaction with the immune system are obstacles that have been identified. Research is ongoing in each of these areas to improve the efficacy/toxicity ratio. Claims of selective magic bullets need to be modest, though, because much remains to be known about the regulation of viral replication and how to harness it.

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Viruses for Treating Cancer

Making viruses into safe and effective agents for treating cancer patients remains a formidable but tantalizing challenge

Matthias Gromeier

Lately, an old idea has recaptured the interests of clinical oncologists and microbiologists alike—namely, to use infectious viruses as an antitumor therapy. The potential of viruses as a cancer therapy was first recognized in cases of unintentional exposure. In 1912, for example, the Italian physician Nicola De Pace reported that cervical carcinomas regressed in patients who had been vaccinated with live, attenuated rabiesvirus. In the following years, other cancer remissions were ascribed to concomitant infections with viruses. These cases most frequently involved hematological malignancies and infections with the measles virus.

Such naturally occurring remissions inspired investigators to study the antitumor effects of experimental virus administration to tumor-bearing animals during the 1920s and thereafter. These experiments involved a wide variety of viruses and often they were tested indiscriminately against tumors of various origin. Documented successes with viral treatment of experimentally induced tumors in laboratory animals eventually culminated in clinical experiments in cancer patients. Uncontrolled trials involving several potentially oncolytic viruses led to some promising responses that were reported in the literature. However, until the modern era, no one conducted rigorously controlled clinical trials of oncolytic viruses.

The antitumor activity of oncolytic viruses is believed, primarily, to reflect tumor cell destruction due to virus infection and intracellular replication

Prospects for Manipulating Pathogenic Properties Are Dramatically Improved

The antitumor activity of oncolytic viruses is believed, primarily, to reflect tumor cell destruction due to virus infection and intracellular replication. However, investigators realized early on that the antitumor effect of viruses might stem not only from direct cytolysis as a result of infection, but also from indirect effects caused by host defense mechanisms provoked by virus replication. This phenomenon became known as "postoncolytic antitumor immunity." Researchers proposed several hypotheses to explain this phenomenon, including (i) immunologic reactions directed against malignant cells elicited by tumor cell lysis, (ii) inflammatory responses to virus infection limiting tumor progress, (iii) interference with replicating preexisting oncogenic viruses in the tumor, and (iv) establishment of noncytolytic infection within the tumor, producing antiviral responses directed against persistently infected tumor cells.

The concept of postoncolytic antitumor immunity relates to much earlier observations by Paul Ehrlich, who produced antitumor immunity in the absence of infection by inoculating experimental animals with tumor tissue lysates. The general principle believed to underlie post-oncolytic antitumor immunity and related concepts evolved into the field of cancer immunotherapy.

Viral oncolysis is again attracting attention, in part because of progress in understanding virus-host interactions and how to manipulate them. Early efforts to produce oncolytic viruses were limited to naturally occurring primary isolates, laboratory strains, and existing attenuated vaccines. Although readily available, these virus preparations were often poorly defined and ill-suited for therapeutic purposes. Now, recombi-

Matthias Gromeier is Assistant Professor in the Department of Molecular Genetics and Microbiology, Duke University Medical Center, Durham, N.C.

nant DNA technology and other techniques enable researchers to manipulate viruses at will, dramatically improving prospects for developing oncolytic viruses into therapeutic agents. Accordingly, following the first description of a genetically modified oncolytic herpesvirus (HSV) by Robert Martuza and his collaborators in 1991, numerous strategies for viral oncolysis have been devised (Table 1).

Viruses proposed for therapeutic use as oncolytic agents. The listed agents are in various stages of preclinical or clinical investigation.

Virus	Genetic Manipulation
DNA viruses	
Adenovirus	
Onyx-015 (dl1520,CI1042)	ΔE1B (55kD)
CN706	PSE(prostate-specific enhancer)-controlled E1A gene
CV787	PSE-controlled E1B gene, probasin-contr. E1A gene
Ad.DF3-E1	MUC1-promoter driven E1A gene
AvE1a04i	AFP (alpha-fetoprotein)-promoter driven E1A gene
Herpesvirus (HSV-1)	
*dl*sptk	Δ thymidine kinase
hrR3	Δ ribonucleotide reductase (ICP6)
R3616, 4009	Δ $\gamma_1$34.5
G207	Δ ribonucleotide reductase (ICP6), Δ$\gamma_1$34.5
NV1020	Δ (derivative of vaccine strain R7020)
G92A	albumin-promoter driven ICP4 gene
Vaccinia	
vv-GMCSF	Δ thymidine kinase, GM-CSF insert
vvDD-GFP	Δ thymidine kinase, Δ VGF
vaccinia virus	
RNA viruses	
Reovirus	None
Vesicular stomatitis virus	None
Picornaviruses	None
poliovirus	IRES exchange with human rhinovirus type 2
coxsackieviruses A15/21	None
Newcastle disease virus	PV701, None

Oncolytic Viruses Need To Overcome Several Hurdles

Nevertheless, converting potentially pathogenic viruses into safe and effective therapies for treating cancer patients remains a formidable challenge. For one thing, viruses interact in complex ways with tumor cells. To reach dispersed loci of invasive or metastatic cancers, such viruses need to penetrate surrounding tissue and evade host defenses before they can target and destroy malignant cells. Moreover, the susceptibility of cancer cells to oncolytic viral agents depends on the presence of appropriate molecules on their surface to which these viruses can bind and use to enter target tumor cells. In addition, oncolytic viruses will need to replicate, meaning this approach will work only in those cases in which tumor cells provide a suitable intracellular milieu. Most importantly, viruses proposed for tumor treatment should be devoid of pathogenic properties that might cause collateral damage to healthy tissue. Combining these properties in a single agent will not be a simple feat.

Researchers are modifying viruses in efforts to meet these requirements, and they are reporting significant progress—developing new ways of administering such viruses to patients, improving their targeting properties, inducing more specific tumor cytolysis, and more fully attenuating the pathogenic properties of some of these viruses.

Virus Tropism: Recognizing the Tumor Targets

To characterize some of the complex interactions between oncolytic agents and tumors, researchers typically first conduct studies in tissue culture or in xenotransplant systems based on clonal tumor cell lines. However, this dependence on tumor cell lines and tissue culture systems has been an Achilles' heel for this field of study. Inevitably, studies in cell culture-based systems are limited because they cannot reproduce key effects that arise from tissue architecture and host defense systems. Although some of these shortcomings can be met by evaluating oncolytic viruses in animal tumor models, other critical questions cannot be addressed through assays based on clonal tumor cell lines alone.

Some of these shortcomings have become evident during the early stages of clinical trials with candidate oncolytic agents based on adenoviruses type 2 or 5. The antineoplastic activity of type 2 and 5 adenoviruses very much depends on a critical attachment factor, the coxsackievirus/adenovirus receptor (CAR). While CAR is not the sole determinant for adenovirus entry into cancer cells, it is essential for conferring susceptibility to this virus. Preclinical tissue culture and xenotransplantation experiments demonstrated efficient cytolysis in cell lines selected

Creatively Melding the Arts and Sciences

A decade ago Matthias Gromeier momentarily thought himself a victim of bad luck when he was forced to veer away from his first scientific passion, HIV, and toward studying poliovirus. But he long since changed his mind. Even as a young medical student, Gromeier says that he felt little interest in practicing medicine. Instead, he was drawn to research, particularly in virology. Not surprisingly, once embarked on studying the poliovirus, he decided to stay with it. "You stick with what works," he says.

A medical student at the University of Hamburg in his native Germany, Gromeier was required to write a thesis before graduating. To do so, he had to find a lab chief willing to take him in. But there were no openings in the AIDS lab, his first choice. "I really wanted to get into HIV, but it wasn't possible," he says. "The only position available was in polio. So I got stuck with the polio virus—and I've never regretted it."

In the years since, Gromeier, 36, who came to the United States in 1993 and is now an assistant professor of microbiology at Duke University Medical Center, has devoted his scientific career to the workings of the poliovirus. More recently, he has focused on the promise of its use in altered form to attack brain cancer. Animal results have been extremely exciting as have lab studies on human cells, and these successes could soon lead to clinical trials.

Motor neurons in the human brain have a receptor, which is an immediate target of the poliovirus, providing a means whereby poliovirus infections can lead to paralysis. Brain tumor cells also have abnormal levels of this receptor, making the malignancies another appealing target for the virus. To ensure that the poliovirus could not attack motor neuron cells, Gromeier and his colleagues remove a sequence encompassing the internal ribosomal entry site (IRES) that the poliovirus needs for making gene products, replacing it with its counterpart from Type II rhinovirus.

"Both are picorna viruses, and very similar in their molecular biology, allowing us to mix and match these genetic elements," Gromeier says. What resulted was "a virus that cannot grow in neurons, but grows perfectly well in tumor cells. Glioma tumors, while they emerge in the brain, are not of neural origin. The virus will grow happily in these tumors—and will kill them in the process."

First Gromeier and his collaborators tested the modified virus in transgenic mice containing the human CD155 receptor gene to determine whether the mice could develop polio-like symptoms. "They didn't get sick," he says. "We had removed its [the virus's] ability to cause disease," he says. Later, the scientists injected this hybrid virus once into mice that were very sick with brain tumors, "and they all recovered within two weeks," Gromeier says. "We had astounding recoveries." Moreover, primary cell lines made from tumors of patients behave much the same. "It works like a charm," he says.

In 1993, he came to this country as a postdoctoral fellow in the lab of Eckard Wimmer at the State University of New York at Stony Brook, where they and their colleagues conducted virus research. He describes Wimmer as "the leading polio virologist—and one of the most renowned virologists—in the world today. It was very attractive for me as a budding scientist to go there." He stayed until 1999, when he moved to Duke University, in Durham, N.C. In September of that year he received the prestigious Burroughs Wellcome Fund Career Award, which provided $540,000 for him to pursue his polio and brain cancer research through August 2003.

Gromeier is the second of three children born to a college economics teacher and his wife, a governess. His younger sister is a medical doctor in Germany, and his older brother is a computer expert who lives in Italy. There was a time when Gromeier thought he would become a musician, rather than a physician who does research in virology. He began playing the cello when he was 8, and took his music very seriously, contemplating a career as a cellist.

"I was quite good, but I wasn't good enough," Gromeier says. "I also couldn't deal with 8-hour practices every day. It's one thing to play for fun, and another to play professionally. I just decided it wasn't for me." He says that although he has not played the instrument in 10 years—"I was burned out"—he is poised to take it up again. He recently had his cello sent from Germany. "I may play in an orchestra here, or in an ensemble, but first I have to start practicing," he says.

Gromeier, who speaks six languages—Italian, French, Spanish, and Portuguese, in addition to German and English—also paints watercolors, with architecture as his exclusive subject, describing it as another pastime that has engaged him since childhood. He spends every weekend painting. Why architecture? "It's what I do best," he says. He believes that the worlds of science and the arts complement each other perfectly. "I think there is a marriage between the two," he says. "You will find many scientists who also have creative careers."

Marlene Cimons

Marlene Cimons is a freelance writer in Bethesda, Md.

for their susceptibility to adenovirus-based oncolytic agents (in other words, cell lines that express enough CAR to permit adenovirus attachment and entry). However, subsequent experiments indicated that CAR expression is erratic among clonal tumor cell lines and even less reliable in tumors occurring in patients.

These and other experiments in tissue culture and xenotransplanted animals indicate that oncolytic viruses can be effective against tumor cell lines without being effective against the target tumor type in general. Indeed, there is a need to understand which molecular determinants of viral oncolysis in cultured cell lines will accurately correlate with those encountered in tumors within patients.

The most obvious susceptibility determinants are cell surface molecules involved in virus attachment and entry. In view of the known discrepancies of expression of cell surface markers in clonal tumor cell lines and primary explant tumor biopsies, virus receptor expression in tumors should be thoroughly characterized before clinically evaluating oncolytic viruses. For instance, histochemical studies could complement expression analyses of homogenized tissues (e.g., Western blot or immunoprecipitation assays) as one means for determining levels of viral attachment proteins within a tumor.

Although desirable, it may be difficult to determine whether individual tumors are susceptible to a proposed oncolytic agent. Moreover, several virus species being proposed as oncolytic agents, including HSV and reoviruses, require multiple cell surface molecules to achieve host cell entry. In the case of the oncolytic type 1 HSV now under clinical investigation, the contributions of several entry mediators to tropism, including HVEM (a member of the tumor necrosis factor family), the poliovirus receptor related molecule 1 (PRR-1, also known as nectin-1), and 3-O-sulfotransferase-3-modified heparan sulfate, may be difficult to unravel. On the other hand, the entry hurdle faced by oncolytic poliovirus recombinants may be comparatively simple. Poliovirus attachment and entry depends on a single entity, the human poliovirus receptor CD155. This simplicity permits investigators to conduct rapid diagnostic tests on tumor biopsy samples. Detecting CD155 may help to predict whether a tumor will be susceptible to oncolytic polioviruses (Fig. 1).

In general, affinity for specific cell surface molecules dictates virus tropism and, hence, is a potent determinant of the oncolytic potential of any virus. To circumvent the limitations of tumor tropism of oncolytic adenoviruses imposed by receptor specificity for CAR, several researchers are altering adenoviruses, giving them affinity for cell surface molecules other than CAR. While changing receptor specificities "by design" may be highly desirable to optimize tumor tropism, it risks changing the pathogenic properties of the virus.

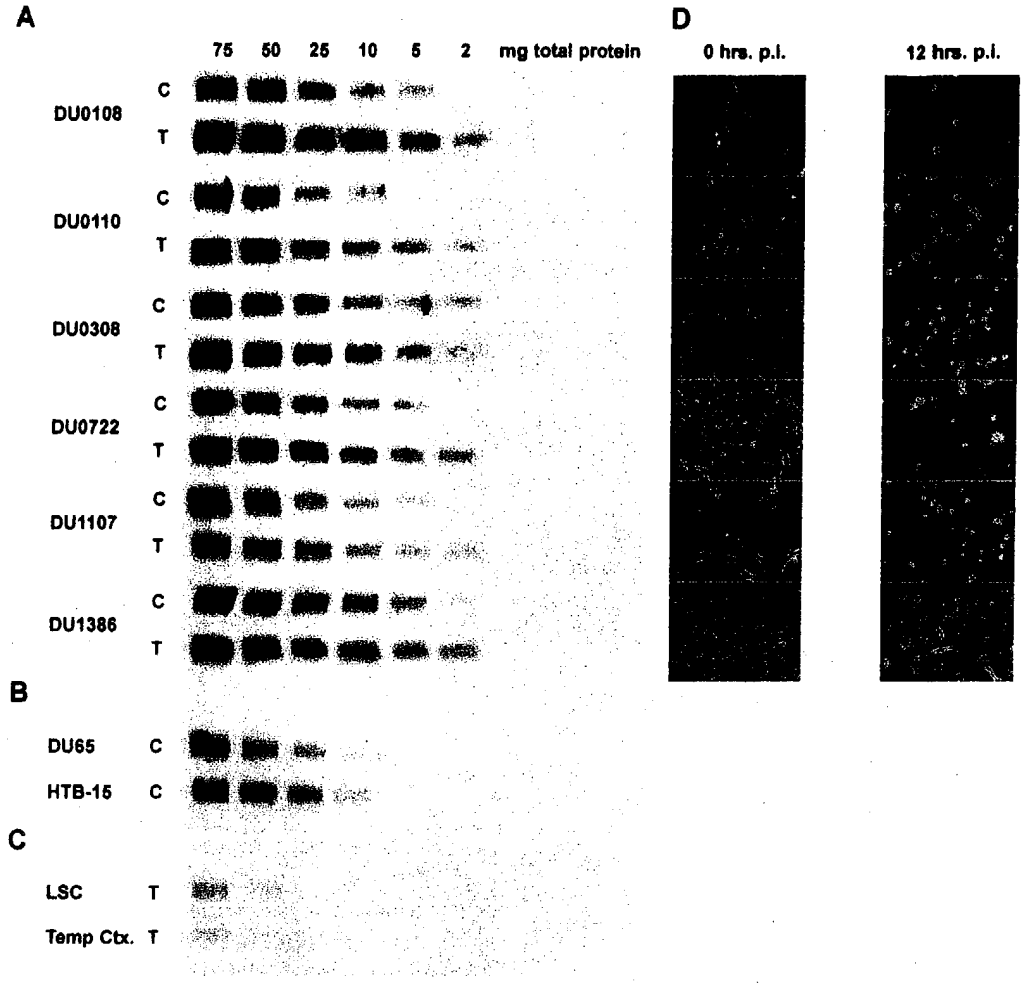
Viruses with a Natural Preference for Cancer Cells

A natural inclination of several virus species towards propagation in transformed cells has been recognized as early as the 1930s. More recent studies have carefully documented the inherent tumor preference of reoviruses, vesicular stomatitis viruses (VSV), and Newcastle disease viruses (NDV) that qualifies these species as oncolytic agents (see table). Tissue culture experiments revealed replication properties of these RNA viruses to markedly improve with malignant transformation of host cells. Although the exact mechanism of inherent conditional replication in malignant cells remains unknown, available evidence suggests defects within host cell responses to viral infection in cancerous cells to account for their sensitivity to these agents.

Interferons (IFN) occupy a central position in the host's defense against viral infection, providing early recognition of the intruder and suppression of virus replication and spread. IFN responses have been linked with a multitude of pathways mediating antiviral activity, either by interfering with specific steps in the virus life cycle or by affecting basic functions of the infected host cell.

Empirical findings stemming from the use of IFNs in cancer therapy revealed that IFN-mediated pathways frequently are defective in malignancy. While nonresponsiveness to IFN generally may confer a growth advantage to cancerous cells, it exposes them to viruses that are readily controlled in nonmalignant cells with intact IFN-mediated defenses. Thus, selectivity for cancerous cells, a prerequisite for clinical application of oncolytic viruses, may be conferred to some virus species through cytogenetic abnormalities in certain tumors. Oncolytic reoviruses

FIGURE 1



Preclinical testing of oncolytic recombinant polioviruses using tumor biopsy material from patients diagnosed with glioblastoma multiforme. **A.–C.** Antigen capture/Western blot analysis of CD155 expression in cell lines (C) and tissues (T) of various origin. Analyses were conducted using decreasing amounts of homogenate (the amount of total protein subjected to the assay is indicated atop). **A.** Low-passage primary explant cell lines derived of glioma biopsy material from brain tumor patients (C) and tumor tissue itself (T) were tested for the presence of the human poliovirus receptor CD155. The assay revealed equivalent levels of CD155 expression in primary explant cell lines and original tumor tissue. **B.** CD155 expression in established glioma cell lines (e.g. DU65 or HTB-15) corresponded to expression levels in primary explant cell lines. **C.** Expression levels of CD155 in the healthy human CNS (lumbar spinal cord=LSC, temp. ctx.=temporal cortex) were exceedingly low. **D.** Viral oncolysis of primary explant glioma cultures derived from patients' tumor biopsies indicates universal susceptibility to oncolytic poliovirus recombinants. Virus treatment resulted in complete lysis of tumor cells 12 hrs post infection (p.i.) in all cases. Efficient viral oncolysis corresponded to the observed universal expression of CD155 in primary explant cell lines and biopsy tissues.

and NDVs are being investigated in clinical trials for activity against a variety of tumor types.

Engineering Viruses Harmful to Tumors but Not the Host

When genetically manipulated, some viruses, including many of those proposed as oncolytic agents (see table), will conditionally replicate in cancerous cells. These include HSVs, adenoviruses, and poliovirus. Without genetic alterations directing virus cytopathogenicity specifically towards tumor cells, therapeutic application of these viruses would be precluded by their pathogenic potential. The concept of conditionally replicating oncolytic viruses was developed with

HSVs and adenoviruses carrying deletions of viral genes. These strategies followed the principle to remove viral gene products that are required for replication in normal tissues but dispensable for virus growth in malignant cell types.

Replication of HSV-1 in natural host cells (i.e., neurons) depends upon a set of gene products involved in nucleotide metabolism (ribonucleotide reductase or thymidine kinase) and neutralization of IFN responses. HSV variants that carry defective ribonucleotide reductase or thymidine kinase genes exhibit attenuation of neurovirulence and fail to induce latency in experimental animals. Similarly, deletion of the $\gamma(1)34.5$ gene exposes the virus to activation of the double-stranded RNA-activated protein kinase (PKR), a critical part of the host defense system against virus infection. Consequently, HSV $\gamma(1)34.5^-$ variants exhibit drastically reduced neurovirulence.

Deletion of these HSV genes, despite their effect on replication in healthy neuronal cell types, does not significantly affect virus replication in rapidly dividing tumor cells. This may be explained by the different intracellular milieu of cancer cells, providing optimal conditions for HSV replication, even in the absence of viral gene products needed in normal cell types. Deleting the $\gamma(1)34.5$ gene appears to generate a situation comparable to viruses with inherent preference for malignant cell types. HSV $\gamma(1)34.5^-$ variants depend on defects within the host cell defense system of tumor cells to unfold selective cytopathogenicity for cancerous cells.

Researchers manipulated adenoviruses to conditionally replicate in tumor cells that express defective forms of p53. For example, they deleted the adenoviral *E1B* gene, whose product has been proposed to be essential for adenovirus replication in normal cells by antagonizing p53. Countering p53 activation may be necessary to prevent cell cycle arrest or apoptosis upon virus infection. On the basis of this hypothesis, investigators postulated that nonmalignant cells, by virtue of expressing functional p53, should block replication of this *E1B* deletion mutant. According to this model, tumor cells with defective p53 would be unable to inhibit replication of the recombinant virus.

However, recent experiments cast doubt on the proposed mechanism of tumor selectivity of *E1B*-deleted adenoviruses, because their replication profiles frequently do not correspond to the p53 status of malignant cells. Extensive experimental evidence indicates that the intricate relation of adenovirus gene expression with the host cell cycle and its importance for virus propagation require careful reevaluation to explain the observed antitumor phenotype of *E1B* deletion mutants.

More recent attempts to achieve selective replication in tumor cells through genetic engineering of viruses include strategies to insert transcriptional control elements that are specifically upregulated in malignant cell types into the viral genome (HSV and adenoviruses). Poliovirus has been manipulated to achieve selective tumor cytopathogenicity at the level of translational control. Although expression of the poliovirus receptor CD155 in malignant brain tumors provides an ideal target for poliovirus (Fig. 1), its inherent neuropathogenic potential does not permit clinical application in brain tumor patients.

To generate a safe oncolytic agent, the natural pathogenic properties of poliovirus had to be eliminated. This challenge was met by manipulating 5' noncoding sequences that are critically involved in translational control of this virus—specifically, the internal ribosomal entry site (IRES). By exchanging the cognate IRES of poliovirus with its counterpart from human rhinovirus type 2 (HRV2), neuropathogenicity is eliminated, based on tests in which this modified virus is inoculated intraspinally in primates.

Although unable to grow in healthy cells of neuronal derivation, these oncolytic polio-/rhinovirus recombinants retain excellent cytolytic properties in malignant gliomas overexpressing CD155 (Fig. 1). Thus, tumor selectivity of oncolytic poliovirus recombinants for CNS tumors, favored by universal receptor expression, is provided at the level of translational control, preventing unwanted neurotoxicity.

Can Viruses Be Harnessed for Molecular Targeting of Tumors?

The obvious advantages of generating conditionally replicating oncolytic agents that respond to specific conditions within malignant cells are offset by the fact that such conditions may be either uncommon or irregularly distributed within tumor tissues. Conditionally replicating oncolytic viruses were characterized in clonal tumor cell lines chosen for their suscepti-

bility to the agent in question. However, clonal cell lines with specific surface characteristics and intracellular properties poorly reflect the varied and irregular appearance of such properties in actual cancers.

There is a marked trend to "individualize" chemotherapeutic cancer treatments, selecting and combining antineoplastic agents according to the characteristics of individual tumors. Because conditionally replicating oncolytic viral agents depend on specific abnormalities within cancer cells, clinical applications of oncolytic viruses may greatly benefit from a similar approach. As with receptor expression, tumors will need to be characterized and specific susceptibility traits detected before treatments begin to help ensure that cancer patients will respond to the particular oncolytic virus being administered.

Can Oncolytic Viruses Be Used Safely in Tumor Patients?

Safety is paramount in developing oncolytic viruses to treat cancers in patients. The inherent pathogenic properties of particular oncolytic viruses may be considered sufficiently mild or may need to be attenuated through genetic manipulation. Several viral species being considered as oncolytic agents, including VSV and NDV, primarily are pathogens of animals that rarely affect humans. Others, e.g., reovirus, are known as "orphans" (the name "reo" is derived from respiratory enteric orphan), viruses that commonly infect humans without causing clinically overt disease.

Frequently, oncolytic viruses based on animal pathogens or orphans are referred to as "nonpathogenic" in humans. This terminology is misleading, in part because they are being called nonpathogenic based on how they behave during naturally occurring infections. However, any therapeutic intervention with oncolytic viruses invariably involves administering them by unnatural routes. Viruses that fail to establish productive infection or elicit clinical symptoms after skin or aerosol exposure may very well do so after being inoculated intravenously, intracerebrally, or intraperitoneally into patients.

Generally, for animal pathogens or orphans, no experimental system is established to evaluate their pathogenicity in nonhuman primates. Therefore, it is very difficult to gauge the immediate consequences and long-term effects of administering such orphan or non-human-pathogenic viruses by invasive means to tumor patients. The situation is further complicated because so many cancer patients, either because of their clinical status or from pretreatments with immunosuppressive drugs, have compromised immune systems.

Proposals for treating patients with oncolytic viruses also sometimes call for using known human pathogens, such as HSV and poliovirus. For both viruses, their neuropathogenic properties need to be attenuated before either one can be considered suitable for therapeutic applications. Fortunately, well-established nonhuman primate models are available for testing whether attenuated oncolytic agents based on either of these viruses are safe.

Similar to other indirect antitumor effects that result from host responses to virus replication within tumors, toxicity may result from immunological or inflammatory responses to such viruses. Reports of severe complications following treatments with viral vectors being tested as gene therapy agents have made the scientific community acutely aware of this problem. These occurrences highlight the need to evaluate—thoroughly and prior to clinical testing—the role of host responses to viruses and other toxicities that might arise if these oncolytic agents were used to treat patients.

The Future of Oncolytic Viruses

Since its conception 100 years ago, the idea of using viruses for treating malignant disease has come a long way. In particular, recent advances in our ability to manipulate viruses as a way of controlling their interactions with the host and with tumor cells is enabling us to harness such agents with advantageous properties, making them safer and more useful in antineoplastic therapy.

Many malignancies targeted by oncolytic viruses respond poorly to available treatments, making the development of alternative treatment regimens an urgent priority. However, this urgency should not seduce virologists, who are designing oncolytic virus-based treatments, and oncologists, who are concerned for their patients, to hasten the testing of candidate therapies that might not be ready for clinical trials. Inadequate preclinical testing can severely re-

tard and even damage the potential success of such clinical applications.

For instance, the dramatic effects seen when using oncolytic viruses to treat tumor xenotransplants in experimental animals often cannot be replicated in patients. Preclinical investigations of oncolytic agents probably will never remove all questions about their efficacy and safety in patients. However, simple assays testing for critical susceptibility determinants such as virus receptors in actual tumors rather than in clonal cell lines are available and can provide a sound basis for moving forward and treating patients with oncolytic viruses (Fig. 1).

The mechanism(s) responsible for tumor cell killing for many oncolytic viruses, including agents being clinically investigated, remain obscure. It is pivotal to determine the role of host responses to virus administration in tumor cell killing ("postoncolytic antitumor immunity" or related concepts) and toxicity. A major incentive for using replicating viruses to treat cancer patients is the ability of such agents to propagate and spread from the initial site throughout cancerous tissues, even to metastatic sites. Proposals seeking to administer oncolytic viruses in very high doses seem misguided, since effectively replicating viruses would produce sufficient titers on their own. Treatment with very high titers of therapeutic viruses might be likely to induce deleterious host responses not related to tumor cell killing.

Rather than developing ever more sophisticated agents exploiting specific abnormalities in clonal tumor cell lines, successful strategies involving oncolytic viruses will depend on correlating the effects achieved in experimental systems with conditions encountered in actual tumors whose properties confer resistance to available treatments.

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